Targeting FOXM1 as a therapeutic strategy in cancer

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Declaration

I, Rebecca Downing, confirm that the research included within this thesis is my own work or that where it has been carried out in collaboration with, or supported by others, that this is duly acknowledged below and my contribution indicated. Previously published material is also acknowledged below.

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Abstract

The transcription factor Forkhead Box Protein M1 (FOXM1) regulates several genes involved in the cell cycle, DNA repair, and oxidative stress, and is frequently upregulated in cancer cells. Whilst targeting FOXM1 alone represents an attractive treatment strategy, emergence of drug resistance makes a combination treatment strategy more clinically relevant.

To investigate novel combination treatments with FOXM1 inhibition, we aimed to exploit the role of FOXM1 in regulating genes involved in the DNA repair pathway, homologous recombination. We hypothesised that reduction of homologous recombination due to FOXM1 inhibition, would render cancer cells synthetically lethal to PARP inhibition. Our data showed the FOXM1 inhibitor Thiostrepton and the PARP inhibitor Olaparib in combination caused greater reduction in cell viability, compared to either agent alone, in breast cancer cells. Mechanistically, our data suggested that RAD51 and BRIP1 may play a role in mediating Olaparib sensitisation upon Thiostrepton treatment, due to increased DNA double-strand breaks in cells treated with our combination therapy.

We also explored the possibility of exploiting the role of FOXM1 in regulating oxidative stress response genes as a combination therapeutic strategy. Our data suggested that FOXM1 and NQO1 may play a role in generating chemoresistance. Furthermore, cancer cells may have a threshold of expression of FOXM1 and NQO1 for viability, where loss of both resulted in the greatest reduction in viability in Ovcar4 ovarian cancer cells. Therapeutically, our data showed for the first time that the FOXM1 inhibitor Thiostrepton and the NQO1 inhibitor Diminutol in combination caused greater reduction in cell viability, compared to either agent alone, in ovarian cancer cells. Our data suggested that our combination therapy may act through different mechanisms, including increased DNA double-strand breaks and increased levels of reactive oxygen species.

Overall, we showed that exploiting the role of FOXM1 as a transcription factor provides a means for therapeutically targeting FOXM1 in combination with drugs that are synthetically lethal with FOXM1 transcriptional target genes.

Abbreviations

2-ME, 2-methoxyestradiaol

ARE, antioxidant response element

BCI, Barts Cancer Institute

BER, base excision repair

Brca1, breast cancer susceptibility gene 1

Brca2, breast cancer susceptibility gene 2

BSA, bovine serum albumin

CTG, Cell-Titer Glo

DCFDA, 2',7' –dichlorofluorescin diacetate

DCIS, ductal carcinoma in situ

DMEM, Dulbecco's Modified Eagles medium

DMSO, dimethyl sulfoxide

DSBs, double-strand breaks

ER, oestrogen receptor

FBS, Fetal Bovine Serum

FOXM1, Forkhead Box Protein M1

HER2, human epidermal growth factor receptor 2

HGSOC, high-grade serous ovarian carcinoma

HR, homologous recombination

IDC, invasive ductal carcinoma

KEAP1, Kelch-like ECH-associated protein 1

LMICs, low and middle income countries

MEFs, mouse embryonic fibroblasts

MM, multiple myeloma

MMR, mismatch repair

NER, nucleotide excision repair

NHEJ, non-homologous end joining

Nqo1, NAD(P)H:quinone oxidoreductase 1

PAPSS1, 3′-phosphoadenosine 5′-phosphosulfate (PAPS) synthase 1

PBS, phosphate buffered saline

PEITC, β-phenylethyl isothiocyanate

PR, progesterone receptor

ROS, reactive oxygen species

RPMI, Roswell Park Memorial Institute medium

SD, standard deviation

SEM, standard error of the mean

siCon, AllStars negative control siRNA

siPLK1, PLK1 siRNA

SRB, sulforhodamine B

SSBs, single-strand breaks

TBHP, Tert-Butyl Hydrogen Peroxide

TCGA, The Cancer Genome Atlas

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Chapter 1

1.0 Introduction to breast cancer

1.1 Breast cancer

Breast cancer is the leading cause of cancer-related death among women worldwide, and it is the most frequently diagnosed cancer [1]. Of the 3.4 million new cases of cancer reported in Europe in 2012, breast cancer was the most common cancer site, accounting for 13.5% of cases [2]. In more recent data, it was suggested that 2.1 million cases worldwide of newly diagnosed female breast cancer cases would be recorded in 2018, accounting for nearly 1 in 4 cancer cases among women [3]. Notably, mammography is the only screening method to have shown reduced breast cancer mortality [4]. In a study that investigated a screening program in London, it was found that mortality risk was reduced by 35% for those attending breast screening at least once [5].

Developments in screening methods, and therefore early detection, have subsequently facilitated an improvement in breast cancer patient survival, in countries that are able to offer this medical support to patients [4]. This needs to be better-developed in low and middle income countries (LMICs) to help in alleviating the burden of breast cancer around the world [4]. These differences, with regard to medical support in LMICs, is further evidenced from the five-year survival rate which is around 85% in the United States and many European countries, while it is around 60% in many LMICs [1, 6].

There are models to assess risk of breast cancer, such as the Gail or Tyrer-Cuzick models [7]. The Gail model assesses multiple risk factors, including age and hormone factors, and it has been validated to be applicable to the general

population [7]. The Tyrer-Cuzick model also assesses classic risk factors, including menopause and body mass index [8]. This model had been suggested to identify few women in the general population at high risk, however the inclusion of mammographic density not only identified women at the extremes of risk distribution, and it may also be valid after evaluation for several years later for follow-up [8]. When considering individual risk factors such as age, it has been demonstrated that breast cancer incidence is low before the age of 30, and this is followed by a linear increase up to 80 years old [9].

Cancers can occur either sporadically, or through an inherited predisposition [10]. Women with mutations in the breast cancer susceptibility genes, *Brca1* and *Brca2*, which are tumour suppressor genes, have a significantly higher risk of developing breast and ovarian cancer, compared to women from the general population [10].

Breast and ovarian cancer risk has been evaluated [10]. Specifically, the Ashkenazi Jewish women population harbors three ancient *Brca1* and *Brca2* mutant alleles, with very few rare family-specific mutations, and has *Brca1* or *Brca2* mutations [10]. Of the observations, by the age of 40 breast cancer risk to relatives with *Brca1* or *Brca2* mutations was 20%, rising to more than 80% by the age of 80 [10]. Furthermore, the lifetime risk of breast cancer among female mutation carriers was 82% [10]. Interestingly, it was also noted that breast cancer onset was significantly delayed in those who had physical exercise and lack of obesity in adolescence [10].

Genetic counselling is routinely offered to individuals at high risk of carrying a *Brca1* or *Brca2* mutation, for the purpose of receiving advice and making decisions regarding their health, such as screening and prevention strategies [11]. In a meta-

analysis study, mean cumulative cancer risks for mutation carriers at age 70 years were determined [11]. For breast cancer risk, it was 57% for *Brca1* and 49% for *Brca2* mutation carriers [11]. Then for ovarian cancer risk it 40% for *Brca1* and 18% for *Brca2* mutation carriers [11]. This type of study was carried out to assist with clinical management and counselling of the at-risk population, to help better-advise patients [11]. Other types of inherited mutation include *PTEN* germline mutations, causing Cowden syndrome, as well as being characterised by a high risk of breast cancer [12]. The lifetime breast cancer risk, for female patients with Cowden syndrome, is 25-50% [12].

1.2 Breast cancer subtypes

Ductal carcinoma in situ (DCIS) is a premalignant proliferation of neoplastic epithelial cells contained within the lumen of mammary ducts, and DCIS can become invasive [13]. It has been suggested that DCIS is a precursor to invasive ductal carcinoma (IDC), and this can culminate in metastatic disease [14]. Whilst IDC is the most common subtype of breast cancer, there is another form of breast cancer known as invasive lobular carcinoma, emphasising the importance of personalised treatment options [15].

DCIS account for approximately 20% of mammographically detected breast cancers [16]. Furthermore, in patients who have had breast-conserving surgery and then experienced local recurrence, half of those have been found to be invasive [13]. Interestingly, it has also been shown that not all high-grade DCIS lesions exhibit a potential for invasion [16].

There are also more specific classifications based on markers, including: oestrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2), and an “intrinsic gene set” has also been identified [17, 18]. Therefore, the intrinsic subtypes that are defined by expression of these markers are luminal types A and B, basal-like and HER2-enriched [18]. Luminal A is ER positive, and/or PR positive, and HER2 negative; luminal B is ER positive, and/or PR positive, and HER2 positive; basal-like is ER negative, PR negative, and HER2 negative; and, HER2-enriched is ER negative, PR negative, and HER2 positive [19].

In a study that investigated breast cancer molecular subtypes, luminal A subtype was identified as the most prevalent, at 34% [20]. This was followed by basal like/triple negative subtype, at 25% [20]. Luminal B and HER2 subtypes had same prevalence, which was 18% each, and there was also breast tissue like/unclassified subtype, which was 5% [20]. In another study, luminal A was also shown to be the most commonly diagnosed subtype, and this subtype had the greatest survival, whereas triple-negative had the poorest survival [21].

Of the classifications, HER2-enriched breast cancer has the highest risk of brain metastases compared to other subtypes, and has been generally identified as having a poor prognosis [22, 23]. There has been another study that showed how triple-negative breast cancer patients have an increased risk of distant recurrence and death, relative to other subtypes [24, 25]. That said, it was determined that triple-negative breast cancer patients who did not show progression after 8 years were then not going to have a recurrence [24]. This differed to other subtypes which could still have a distant recurrence up to 17 years after diagnosis [24].

The ability to determine the subtype of a breast cancer patient is important for guiding treatment option decisions [26]. Not only does it guide more personalised treatment decisions, it has also been shown to be important in deciding which patients would need to have preoperative breast magnetic resonance imaging [26]. Specifically, this magnetic resonance imaging can help to detect characteristics such as lymph node involvement in luminal B and HER2 molecular subtype breast cancers, where these patients are more likely to have clinically significant disease, thus changes in treatment strategies may affect clinical outcomes [26].

1.3 Current treatment options for breast cancer patients

1.3.1 Early treatments and targeted treatments

The majority of breast cancers diagnosed in early stages are offered surgery, which can be as a mastectomy or lumpectomy [27]. Breast-conserving surgery comprises of a lumpectomy, and it is also important to have some normal tissue removed to indicate the cancer has been excised [27]. In a study where radical mastectomy was compared with less extensive surgery, it was found that there was no advantage to having radical mastectomy, and that less radical surgery seemed just as effective [28]. Furthermore, in another study of breast cancer patients who had undergone either radical mastectomy or breast-conserving surgery, the long-term survival rate among women was found to be the same regardless of their surgery type [29].

For early stage breast cancers, complete resection by surgery can remove the cancer, but there is a chance that over time there may be a recurrence [30].

Therefore, surgery followed by adjuvant treatment offers patients a more effective treatment option, which could also be considered alongside neoadjuvant treatment [30]. Breast conservation is today's intended surgical standard of care for most breast cancer patients, to avoid what would have previously been a primary mastectomy, and this is supported by advances in oncoplastic surgical techniques and successes of neoadjuvant tumour-shrinking drug therapies [31].

As illustrated in Figure 1, there are a number of systemic ways to treat early-stage breast cancer. Once the breast cancer subtype has been determined, based on this information, the most appropriate treatment can be offered to the patient. To support an improved prediction of clinical outcome, the 70-gene signature was developed, alongside other gene-expression profiles, and this has proven to be particularly valuable in recognising which patients may benefit from adjuvant chemotherapy [32, 33]. The 70-gene signature classifies tumours into groups that are associated with a good prognosis or a poor prognosis, and this is on the basis of the risk of distant recurrence at 5- and 10-years [32, 33].

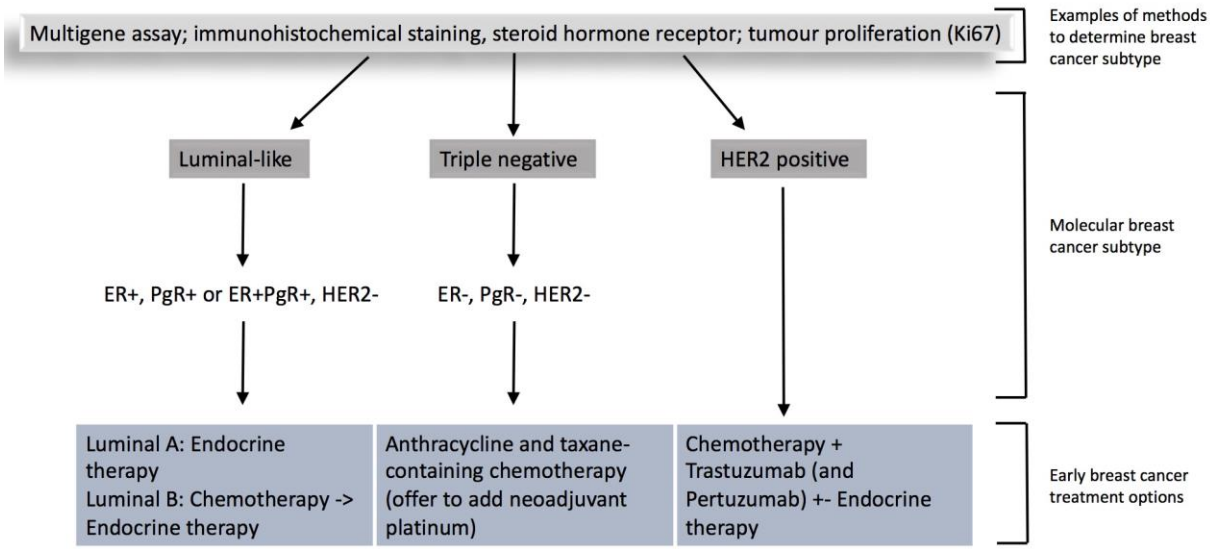


Figure 1. Treating early breast cancer.

There are a number of systemic ways to treat early breast cancer, depending upon the molecular breast cancer subtype of the patient (adapted from: [31]).

Breast cancer patients are often over-treated and exposed to the toxicity of chemotherapy, without benefits, therefore this could be avoided by incorporating the 70-gene signature into clinical decisions [33]. In fact, more than 30 different signatures have been reported [34]. As breast cancer is heterogeneous, it has been suggested that there may not be a one-size-fits-all signature; however these authors did report that better models could be built from common genes across different signatures [34].

The characteristics considered for treatment decisions is not an exhaustive list, however, such that tumour characteristics including tumour grade and size are also important [33, 35]. Furthermore, patient characteristics including age and menopausal status provide additional information in making an informed decision [33, 35]. Whilst not accounting for individual biologic characteristics of patient

tumours, tools like PREDICT Plus are also available to assist in treatment option choices [33].

Tamoxifen is an ER antagonist that has been used to treat patients with ER-positive breast cancer [36, 37]. However, aromatase inhibitors such as Letrozole have been shown to be a more effective endocrine therapy in the treatment of breast cancer patients, than Tamoxifen [38]. Aromatase inhibitors work by indirectly affecting ER function, through blocking the conversion of adrenal androgens to oestrogen in the peripheral tissues of postmenopausal women, including the breast [38].

For patients with metastatic breast cancer and a germline *Brca* mutation, the FDA-approved oral PARP inhibitor Olaparib has been shown to have promising activity, over standard therapy [39]. This comes after Olaparib has already been used to give notable clinical improvements in the treatment of patients with recurrent ovarian cancer and a *Brca* mutation [40, 41].

HER2 overexpression and/or amplification have been shown in around 25% of breast cancer cases [42, 43]. The targeted therapy known as Trastuzumab, a humanised monoclonal antibody against the extracellular domain of HER2 has benefitted patients with HER2-positive breast cancer, alone and in combination with chemotherapy, as well as following adjuvant chemotherapy [43-45]. More recently, another humanised monoclonal antibody against HER2 known as Pertuzumab, which binds different domains of HER2 compared to Trastuzumab, has been shown to be effective in a treatment strategy which also includes Trastuzumab and chemotherapy in HER-positive breast cancer [46].

2.0 Introduction to ovarian cancer

2.1 Ovarian cancer

Ovarian cancer is the seventh most commonly diagnosed cancer among women in the world [2, 47]. It is the second most frequent gynaecologic malignancy, after endometrial cancer, and about two thirds of tumours occur at reproductive age [48]. In comparison to other female genital cancers, ovarian cancer carries a higher mortality rate [48].

No singular diagnosis exists for ovarian cancer, such that many of these tumours involve the ovary secondarily, thus originating from other gynaecological tissues [47]. A gene profiling study showed that there was a significant correlation between gene expression in, for example, the fallopian tube mucosa and in serous carcinomas [49]. The prevailing view for the origin of high-grade serous ovarian carcinoma (HGSOC) was that it developed from the ovarian surface epithelium, however this had never been shown to be reproducible [50]. A study was therefore carried out to investigate the origin of HGSOC, and it was found to originate in the fallopian tube [50]. In fact, <1.25% of HGSOC are confined to the ovary at diagnosis, so this type of study can help improve clinical practice to carry out systematic sectioning and examination of the fallopian tube [50].

Many patients present with advanced-stage disease, and this is because it is asymptomatic at the early stages, making detection more difficult [51]. There is no bleeding or obvious lump, and the types of symptoms that could be expected include bloating and frequent urination [52]. The 5-year survival rate is usually less

than 20% because often dissemination has already occurred when a patient is diagnosed [53].

Unfortunately, screening methods in ovarian cancer have not been very successful and, if screening was to be used, one screening test would not be effective at detecting all types of ovarian cancer. For Type I ovarian carcinomas, these could be potentially more easily detected by pelvic examination and/or transvaginal ultrasound [54]. Then for Type II ovarian cancers, these become more difficult to detect because these tumours are not always confined to the ovary at diagnosis [54]. This could be improved by determining more sensitive and specific biomarkers for detection [54]. Furthermore, clinical trials are being carried out to investigate the efficacy and cost-effectiveness of screening for ovarian cancer [55].

Specific biological causes of infertility, such as endometriosis, have been linked to increased risk of ovarian cancer [56]. Furthermore, accumulating evidence has linked the benign disease endometriosis as a precursor to endometriosis-associated ovarian cancer, in particular the endometrioid and clear cell subtypes [53]. Women who have used oestrogen-only replacement therapy, especially those that have used it for 10 years plus, have also been shown to have an increased risk of ovarian cancer [57]. In another study, it was found that older women, as well as women who had never used oral contraceptives, had an increased risk of rapidly fatal disease [58]. In this study, for example, the authors account for certain limitations such as not knowing the grade of all women, and this highlights the continued need for further study in this area [58].

As discussed in the introduction to breast cancer, *Brca1* and *Brca2* mutations not only increase the risk of breast cancer, but these mutations also increase the risk of ovarian cancer. An inherited mutation in *Brca1* or *Brca2* accounts for around 10% of invasive ovarian carcinomas, with an even greater prevalence in Ashkenazi Jewish women, which is closer to 50% [59]. In a study where the prevalence of homologous recombination (HR)-related gene mutations was investigated across multiple cancers, ovarian cancer featured among the most commonly mutated lineages, accounting for a frequency of 20% [60]. While it has been suggested that BRCA-associated and sporadic ovarian carcinomas seem to have similar histopathologic characteristics, p53 mutations have a higher frequency in hereditary ovarian carcinomas [59]. Interestingly, a study showed that when comparing advanced-stage BRCA-associated ovarian carcinoma patients with sporadic ovarian carcinoma patients, there was a significantly improved survival [59]. This type of study can help clinicians with decisions regarding patient treatment options, and it can also help in assessing patient suitability for clinical trials [59].

2.2 Ovarian cancer subtypes

There are two broad categories used to divide ovarian cancer, Type I and Type II, and these are subsequently made up of several subtypes [51]. They are characterised by individual genes as well as certain molecular signalling pathways [54].

Type I carcinomas include endometrioid, clear cell, mucinous and low-grade serous, and these are slow-growing indolent neoplasms, with clearly described precursor lesions [51]. Type II carcinomas includes high-grade serous, and these are clinically aggressive neoplasms and may develop de novo from the tubal and/or ovarian

surface epithelium [51]. Serous carcinomas are the most common type and high-grade makes up about 70% of ovarian carcinomas [61]. The genes that characterise the high-grade serous subtype, for example, are p53 mutation and *Brca1/2* inactivation [54]. Furthermore, constitutive activation of molecular signalling pathways, such as cellular proliferation, can occur through mutated gene products [54].

Type I ovarian cancers, accounting for 5-10% of all epithelial tumours, are typically *KRAS*, *PIK3CA*, *PTEN*, or *BRAF* mutated, and often diagnosed at the early stages [62]. Type II tumours account for 90% of deaths from ovarian cancer due to late diagnosis in the advanced stage and are frequently *p53* mutated [62]. A mutation in the tumour suppressor *p53* is considered a hallmark of high-grade serous ovarian cancer, present in nearly 100% of cases, compared to Type I carcinomas, where *p53* is rarely mutated [62].

Interestingly, in a study where p53 status was analysed in high-grade serous ovarian carcinomas, it was discovered that patients who had wild-type p53 were seemingly more chemoresistant and had a poorer survival, compared to patients who had mutated p53 [63]. Whilst the mechanism was not clear in this study, a mouse model had previously shown that wild-type p53 promoted ovarian cancer cell survival and invasion [63, 64].

Ovarian clear cell carcinoma accounts for <5% of all ovarian malignancies, and a study showed that complete surgical staging, which included pelvic and para-aortic lymphadenectomy and chemotherapy, Paclitaxel plus Carboplatin, was able to improve survival of patients with ovarian clear cell carcinoma, stage I [65, 66]. This

type of study can benefit a large amount of patients, as up to 60% of patients have stage I disease at the time of diagnosis [65].

Metastasis of ovarian carcinomas is rarely through the vasculature, and the most common sites of metastasis include the fallopian tube and contralateral ovary [67]. It has been suggested that metastasis is the cause of bilateral ovarian cancer, whereby the spreading to the contralateral ovary seems to be a late event in the clonal evolution of these cancers [68].

2.3 Current treatment options for ovarian cancer patients

2.3.1 Early treatments and targeted treatments

Over the last two decades, surgery and chemotherapy as a primary treatment have had minimally improved results, and progression-free survival has been maintained at around 18 months [69]. Around 30% of ovarian cancer patients are diagnosed at early-stage disease and receive surgery, however it has been suggested that recurrence will happen in 10-50% of these patients [70]. It has been shown that adjuvant chemotherapy used in patients with early-stage disease does cause a delay in disease recurrence [70].

Furthermore, patients with advanced ovarian cancer can also go on to tumour progression or recurrence, and the added risks associated with this include drug resistance [69]. In cases where there is a high chance of gross residual disease after primary debulking surgery, the use of neoadjuvant chemotherapy can help to

reduce tumour load [71]. It is also interesting to note that time interval between surgery and adjuvant chemotherapy can affect the growth of metastasis, and one study has shown that early initiation of chemotherapy is important to improve overall survival of ovarian cancer patients [72]. Then there was a 4% decrease in relative overall survival when adjuvant chemotherapy was delayed, each week [72].

Some of the early treatment options for patients with different subtypes of ovarian cancer are listed in Figure 2. When considering the subtype of mucinous ovarian cancer, one study has shown that the response to first-line platinum-based chemotherapy is worse compared to other subtypes, and it has been suggested that fluorouracil-based chemotherapy may be a better alternative because of its use in treating mucinous gastrointestinal carcinomas [73]. Another study has shown that recurrent mucinous ovarian cancer also has a low response to chemotherapy [74].

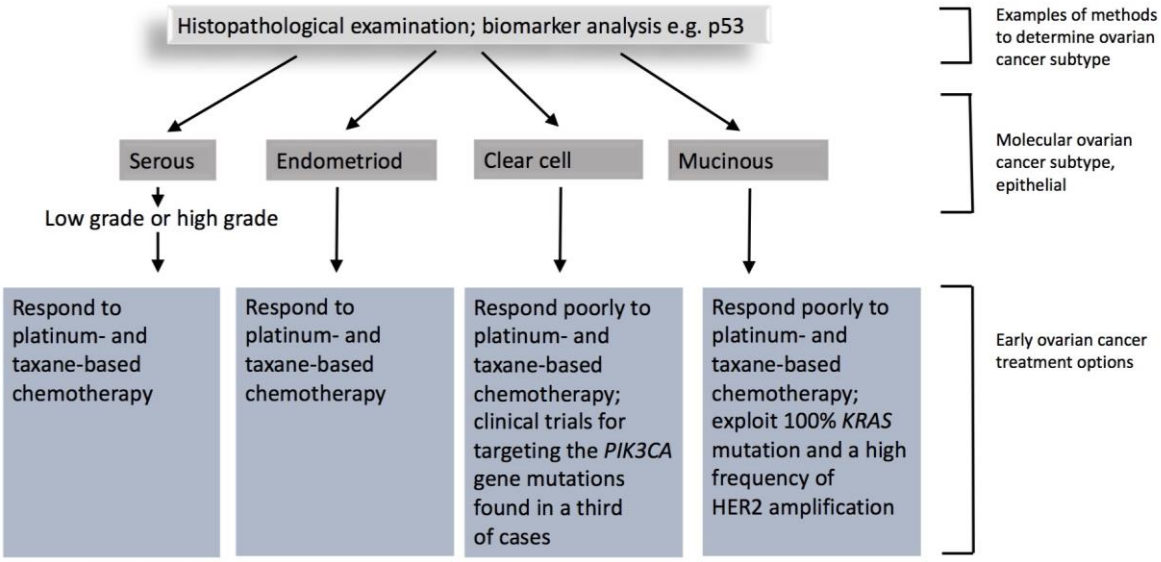


Figure 2. Treating early ovarian cancer.

There are a number of systemic ways to treat early ovarian cancer, depending upon the molecular ovarian cancer subtype of the patient (adapted from: [75, 76]).

Cisplatin was the first FDA-approved platinum compound for cancer treatment in 1978, and this chemotherapy acts through binding DNA to cause DNA damage which blocks cell division and leads to apoptosis [77]. Drug resistance and side effects are always important considerations in the treatment of cancer patients, so this led to the development of analogues of Cisplatin, and Carboplatin was subsequently identified and shown to have fewer side effects than Cisplatin [77-79]. That said hypersensitivity reactions have been found to be an issue in recurrent ovarian cancer patients who have been challenged again with Carboplatin, where a discontinued platinum-based therapy schedule could impact on a patient's prognosis [80]. Therefore, a study was able to show that hypersensitive-related risks could be reduced, and the benefits of platinum could be maintained, by treating these patients with Cisplatin [80].

Paclitaxel is another chemotherapeutic compound introduced in the 1990s, and it showed less toxicity and was easier to administer when used to treat patients in combination with Carboplatin, rather than in combination with Cisplatin [79, 81]. A study was carried out to investigate the schedule of treatment for patients with ovarian cancer, and whether patients would benefit from weekly Paclitaxel, compared to every-3-weeks Paclitaxel [82]. Interestingly, the weekly schedule did not prolong progression-free survival among patients with ovarian cancer, in comparison to having Paclitaxel administered every 3 weeks [82]. These types of studies are important to gain a more in-depth understanding of managing the costs associated with different treatment regimens for ovarian cancer patients [82].

The PARP inhibitor Olaparib is recognised as being the first compound to be approved for ovarian cancer treatment since 1996, illustrating the challenges in

developing novel treatments for these patients [40]. This treatment is offered to patients with deleterious or suspected deleterious germline *Brca*-mutated advanced ovarian cancer, and it has been shown to be effective as a monotherapy option in patients who have received three or more prior lines of therapy [40, 41]. Another study has shown that there may be other clinically relevant populations who can derive benefit from Olaparib, similar to patients with *Brca*-mutated ovarian cancer, where the tumours in these patients have loss-of-function mutations in other HR genes [83]. Genes involved in more than one patient included *Brip1* [83].

3.0 FOXM1 plays a role in normal cellular events and cancer

3.1 Introduction to FOXM1

3.1.1 FOXM1 is a transcription factor

Forkhead Box Protein M1 (FOXM1) (also known as Trident, MPP2, WIN, or HFH-11) is a transcription factor that belongs to the Forkhead box family of proteins [84, 85]. This family of transcription factors, one of the largest classes of transcription factors in humans, are defined by a conserved winged helix DNA binding domain, and spans a wide range of species [85, 86]. Expression of FOXM1 is found in all proliferating cells and it is required for cell division, however expression is absent in quiescent and terminally differentiated cells [85, 87].

3.1.2 Structure and isoforms of FOXM1

Outside of their conserved DNA binding domain, the structure of the Forkhead box proteins differ, such that FOXM1 also contains an N-terminal repressor domain and a transactivation domain [88]. FOXM1 is made up of 10 exons, and three distinct variants are formed through alternative splicing: FOXM1a, -b, and -c [84, 89] (Figure 3). More recently, a fourth isoform has also been identified, known as FOXM1d [90].

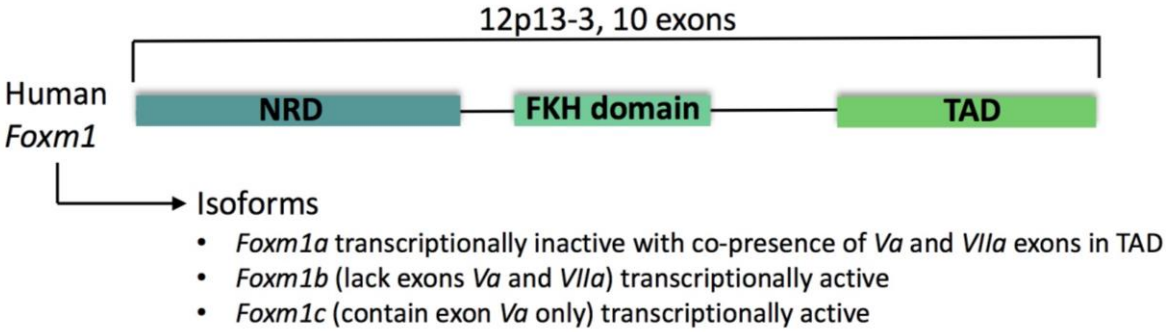


Figure 3. Structure of FOXM1.

The N-terminal repressor domain (NRD), the Forkhead (FKH) domain, and the transactivation domain (TAD) can be alternatively spliced to give rise to three isoforms: FOXM1a, -b, and -c (adapted from: [88, 91]).

The FOXM1a isoform is transcriptionally inactive because of disruption to its transactivation domain, while the FOXM1b and FOXM1c isoforms are both identified as being transcriptionally active, without this disruption [87, 92]. FOXM1a is not conserved in mouse, which makes it more difficult to determine the physiological significance of this isoform [93]. Interestingly, the FOXM1a variant may act as a dominant-negative variant because of retaining normal DNA binding activity, without a functional transactivation domain [94]. FOXM1a could compete with other splice variants to bind FOXM1-responsive elements, and inducing this variant could reduce cell proliferation caused by FOXM1b and FOXM1c [94].

Transcriptional activity of FOXM1 is prevented by an association of the N-terminal domain of FOXM1 with the C-terminal activation domain [95]. It was also shown that when the N-terminal domain was deleted, a constitutively active transcription factor was generated and functioned independently of phosphorylation events [95]. Furthermore, these cells exhibited increased transforming activity [95].

3.1.3 Localisation and regulation of FOXM1

Localisation of FOXM1 appears to be cell cycle-dependent, such that FOXM1 is localised mainly to the cytoplasm in cells at late-G1- and S- phases [93]. Just before entering G2-/M- phase, FOXM1 is then translocated to the nucleus, and this is when FOXM1 is phosphorylated, such that activated Raf/MEK/MAPK signalling was found to promote FOXM1 phosphorylation [93]. Importantly, this group also provided the first evidence that Raf/MEK/MAPK signalling and FOXM1c mediate cell cycle progression through G2/M [93]. Further evidence supports that FOXM1c is in fact a greatly attenuated form of FOXM1, and it requires Raf/MEK/MAPK signalling for activation [96]. Whilst the b isoform represents a more active form of FOXM1, and has been frequently associated with overexpression in cancer cells [96]. Indeed, FOXM1b has been suggested to be a potent activator of tumour metastasis [97].

During the cell cycle, FOXM1 itself undergoes regulation through post-translational modifications, including the direct phosphorylation by PLK1 which leads to activation of FOXM1's transcriptional activity [98]. It is these phosphorylation events that have been suggested to relieve the repressive function of the N-terminal domain in FOXM1 [99]. That said, more recent evidence has suggested that SUMOylation is more likely blocking FOXM1 dimer formation, to alleviate autorepression of FOXM1 [100]. This modification has been shown to peak in G2-

and M-phase, and this Small Ubiquitin-Like Modifier-2 (SUMO-2) protein has been suggested to control FOXM1 transcriptional activity [100].

It was observed that there is a progressive decrease in FOXM1 mobility when cells were released from G0/G1 arrest, and that there is an initial phosphorylation of FOXM1 in S-phase which leads to hyperphosphorylation in G2/M, when PLK1 activity is at its highest [98]. So FOXM1 has been shown to be phosphorylated in the carboxy-terminal domain by Cdk1 initially, and this acts as a priming phosphorylation, ready for subsequent PLK1 phosphorylation [98]. Furthermore, it is FOXM1's hyperphosphorylation during G2/M phase that correlates with its transcriptional activity being cell-cycle-restricted [98].

To understand why phosphorylation and activation of FOXM1 are prevented at earlier stages, and restricted to G2, there was additional investigation of interactors with FOXM1. B55α, a regulatory subunit of the protein phosphatase 2A (PP2A), was subsequently identified as a novel interactor of FOXM1 [101]. The phosphatase PP2A/B55α has been shown to counteract phosphorylation of FOXM1, and negatively regulate the activity of FOXM1 [101]. It was suggested that PP2A/B55α might counteract indirectly the activation of FOXM1 by PLK1 [101].

FOXM1 is also regulated by the E3 ubiquitin ligase, with its substrate specificity subunit, APC/C-Cdh1, a complex that participates in proteolysis [102, 103]. This happens in late M-/early G-phase of the cell cycle and it is involved in regulating entry into S-phase [102, 103]. It has been suggested that Cdh1 recognises FOXM1 as a target, by the degradation motifs that are present in FOXM1's N-terminal domain [102]. As previously mentioned, the N-terminal domain acts as a

transcriptional repressor through intramolecular interaction with the C-terminal domain [95], therefore the role of this domain in degradation further emphasises its importance in tight control over the activity of FOXM1 to the G2-phase in G2-specific gene expression [102]. Furthermore, another study showed how FOXM1 is downregulated by p53 after DNA damage, to lead to a stable G2 arrest and maintenance of genomic integrity [104].

3.2 Role of FOXM1 in the cell

3.2.1 Cell cycle

From S-phase, FOXM1 expression levels become increased and these levels persist until the end of mitosis, where the function of FOXM1 is important for proper mitotic progression [105]. This has been illustrated by FOXM1's role in regulating a large number of G2-/M-specific genes, as well as FOXM1-mediated transactivation of cyclin B for timely mitotic entry [105, 106]. In another study, FOXM1 was found to be a requirement for cells to avoid mitotic catastrophe [106]. As FOXM1 plays a diverse role in cell cycle regulation, this has been summarised in Figure 4, indicating where FOXM1 is acting on the cell cycle.

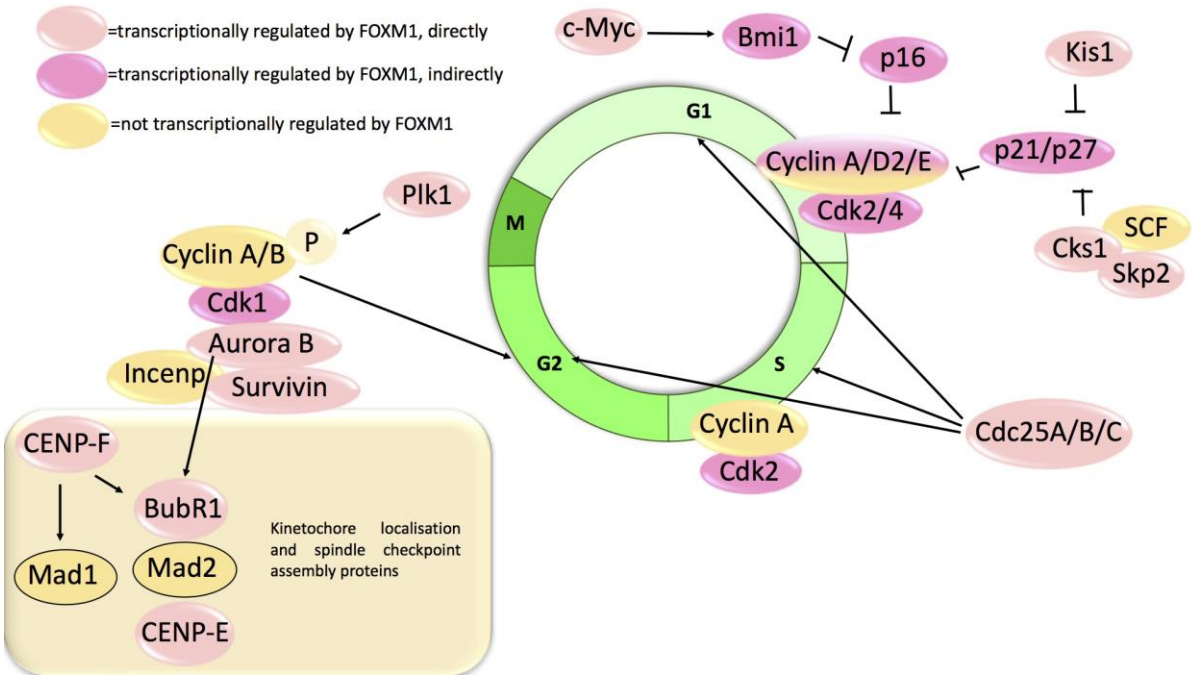


Figure 4. FOXM1 plays a role in the cell cycle.

A number of proteins involved in the cell cycle are either directly or indirectly transcriptionally regulated by FOXM1 (adapted from: [88, 107]).

Briefly, one of the roles of FOXM1 is to regulate *Skp2* and *Cks1*, to then target cyclin-dependent kinase inhibitors for degradation during G1-/S- phase transition [108]. The SKP2 and CKS1 proteins are specificity subunits of the SCF ubiquitin ligase complex, and these are essential for the recognition of the phosphorylated $p27^{KIP1}$ and $p21^{Cip1}$ proteins [108]. The degradation of cyclin-dependent kinase inhibitors subsequently prevents Cdk2-cyclin E complex inhibition so that RB protein is phosphorylated and ultimately stimulates genes for S-phase progression [108]. Another role of FOXM1 is to activate the transcription of Cdc25b, which is essential for the activation of the Cdk1-cyclin-B complex, during the G2/M transition [109]. Cdc25b is a phosphatase that dephosphorylates Cdk1 to promote M-phase progression, thus providing a mitotic checkpoint through regulating Cdk1/cyclin B kinase activity [109].

3.2.2 Differentiation, migration and metabolism

Functions of FOXM1 are not limited to cell cycle regulation. In one study, *Foxm1*$^{-/-}$ embryos were shown to survive until late gestation, with numerous well-developed embryonic tissues [110]. These tissues showed no proliferation defects [110]. During embryonic lung development, the role of FOXM1 in differentiation has been further explored, such that FOXM1-deficient mesenchyme has been shown to be unable to differentiate into capillary endothelial cells [111].

Other roles of FOXM1 have been investigated using different models, including cancer cells. In one study using U2OS osteosarcoma cells, FOXM1 was shown to directly activate *JNK1* transcription [112]. This protein was not only shown to contribute to FOXM1-regulated G1/S transition, but also tumour cell migration, invasion, and anchorage-independent growth [112]. Furthermore, in a study using pancreatic cells, the authors were able to show differences between isoforms of FOXM1, such that FOXM1b and FOXM1c overexpression promoted pancreatic cancer cell migration and invasion, but not FOXM1a [92]. Additionally, FOXM1c was found to be the predominant isoform in pancreatic cancer cells, suggesting more of a role for this isoform in pancreatic cancer development and progression [92]. While FOXM1b, along with FOXM1c, may play more of a role in pancreatic cancer cell biology [92].

In another study using pancreatic cells, FOXM1 was implicated in regulating glucose metabolism via transactivation of LDHA expression [113]. Then, in a later study using epithelial ovarian cancer cells, FOXM1 was again shown to regulate glucose metabolism, this time through direct activation of the glycolytic genes glucose transporter 1 (GLUT1) and hexokinase 2 (HK2) [114]. FOXM1 expression

was positively correlated with GLUT1 and HK2 expression in epithelial ovarian cancer cells [114]. This group also found that inhibition of FOXM1 expression by shRNA did not affect expression of LDHA in epithelial ovarian cancer cells, and therefore different mechanisms may be acting across different cancer types [114].

3.2.3 DNA damage response

In FOXM1-deficient cells, it was observed that there are increased DNA breaks, illustrating an integral role for FOXM1 in the regulation of genes involved in DNA repair [115]. Beyond avoiding the accumulation of DNA breaks, FOXM1 has been recognised to be instrumental in the transcriptional regulation of a number of DNA repair genes, including *Brca2* and *Xrcc1* [115]. This study also demonstrates how FOXM1 is involved in regulating different DNA repair pathways, including homologous recombination (BRCA2) and base excision repair (XRCC1) [115]. In addition, DNA damage in p53-depleted cells was shown to increase FOXM1 expression levels, therefore supporting this data that DNA damage positively regulates FOXM1 protein stability [104, 115].

Further studies of homologous recombination have identified FOXM1 as a transcriptional regulator of the genes *Rad51*, *Brip1* and *Nbs1* [116-118]. When a microarray analysis was performed on FOXM1-depleted cells, *Rad51* was noted as a potential downstream target of FOXM1 [116]. This was confirmed in stable cell lines of FOXM1-depletion, and it was subsequently found that FOXM1 directly binds to the promoter of *Rad51* to regulate its activity [116]. This study also suggested that the FOXM1-RAD51 axis is involved in chemotherapy resistance [116].

A link was also found between FOXM1 and BRIP1 [117]. In their model, the authors were able to demonstrate that *Foxm1* silencing resulted in decreased expression of BRIP1 [117]. Consistently, *Foxm1*$^{-/-}$ mouse embryonic fibroblasts (MEFs) showed lower BRIP1 expression levels compared to WT MEFs [117]. The authors also established *Brip1* as a direct transcriptional target of FOXM1 [117]. In addition, FOXM1's role in regulating the expression of DNA repair genes, as in this instance with *Brip1*, has also been implicated in playing a role in drug resistance [117].

In the study of NBS1, whilst FOXM1 was shown to directly regulate *Nbs1* expression, other components of the MRN complex, MRE11 and RAD50, were found not to be under direct regulation of FOXM1 [118]. They were instead suggested to have their protein levels stabilised through forming an active MRN complex [118]. This also indicates that NBS1 may be limiting for the formation of the MRN complex and that its level could set the threshold for DNA repair activity [118]. Furthermore, upon FOXM1 overexpression and NBS1-depletion, HR activity was not increased, so NBS1 is essential to FOXM1's HR function [118]. It was also suggested that the regulation of NBS1 by FOXM1 contributes to the DNA damage response, genotoxic drug resistance and DNA damage-induced senescence [118].

3.2.4 Oxidative stress response

One of the main studies that investigated FOXM1 and oxidative stress showed how FOXM1 plays a role in regulating oxidative stress in the context of oncogenesis [119]. FOXM1 has also been shown to counteract oxidative stress-induced premature senescence, through regulating Bmi-1 expression [120].

It was shown that cells deficient in FOXM1 showed increased levels of ROS compared to normal cells, thus FOXM1 was shown to be an important regulator of the levels of intracellular ROS [119]. FOXM1 was also shown to bind to MnSOD and catalase promoter regions, further emphasising the role of FOXM1 in regulating oxidative stress through the regulation of anti-oxidant genes [119]. The authors then went on to speculate a possible role for FOXM1 in attenuating apoptosis, where mitochondrial ROS is involved, by upregulating detoxifying enzymes such as MnSOD [119]. Overall, it could be that tumour cells use FOXM1 to control oxidative stress and this is how premature senescence and apoptosis can be escaped [119].

In a later study, normal cells and cancer cells were both identified to use FOXM1 as a key regulator of ROS levels and oxidative stress [121]. Normal keratinocytes and head and neck squamous carcinoma cells were sensitised to apoptosis when depleted of FOXM1 [121]. Again, as inferred by Park et al., these authors suggest that cancer cells expressing high levels of FOXM1 could be exploiting the anti-oxidant activity of FOXM1 so that cancer cells escape premature senescence and apoptosis [121]. Furthermore, in a study of human embryonic stem cells, expression of FOXM1 was shown to be enriched and regulated in a cell cycle-dependent manner, and when FOXM1 was depleted in these cells they were sensitised to oxidative stress [122]. Interestingly, when the authors investigated the previously reported transcriptional targets of FOXM1, MnSOD and catalase, in FOXM1-depleted human embryonic stem cells, they only observed a decrease in the expression of catalase and not MnSOD [122]. Therefore, FOXM1-depleted human embryonic stem cells may be sensitised to oxidative stress through reduced catalase expression [122].

3.2.4.1 Oxidative stress in cancer

Reactive oxygen species, at the right concentrations, play an important role in signalling processes [123]. For instance, an ‘oxidative burst’ has been described to occur in the first line of defence against pathogens, where ROS is produced by phagocytic NADPH oxidase [123]. Indeed, mice deficient in the gp91 protein of reduced nicotinamide adenine dinucleotide phosphate oxidase have been shown to have increased susceptibility to Listeria infection, for example [124].

It is only when these concentrations of ROS become even higher than required, and this can be excessive and/or a sustained increase, then damage to the cell can lead to disease such as cancer [123]. Oxidative stress contributes to cancer in a number of ways; impacting on redox state, driving tumour progression, and adapting to hypoxic conditions [123, 125, 126].

In patients with advanced malignancies a pro-oxidative shift in the plasma thiol/disulfide redox state has been observed, termed “mitochondrial oxidative stress”, suggested to be linked to uncontrolled availability of mitochondrial energy substrate [123]. Furthermore, higher levels of H_2O_2 and NADPH oxidases (NOXs) have been shown to play a role in increased tumourigenicity and angiogenesis in various cancers, including prostate cancer [125].

Cancer cells also promote HIF-1α and HIF-2α accumulation, leading to transactivation of their target genes involved in processes like angiogenesis and metabolic adaptation to hypoxia, where ROS has been shown to stabilise HIF-1α protein in an AMPK-dependent manner [126]. AMPK plays a critical role as an energy sensor in response to stresses, such as hypoxia and ROS, and this study

showed that AMPK was required for ROS-induced HIF-1α induction, stabilisation, and transcriptional activation [126]. Whilst, under hypoxic conditions, AMPK activity was only involved in HIF-1 transcriptional activation, identifying AMPK as a key determinant of HIF-1 functions in response to ROS [126].

Activation of oncogenes is a key step in cellular transformation and maintenance of cancer, where an increase in ROS accumulation has also been reported in these cells [127]. Oncogenes, including Ras and Myc, give rise to compromised genome integrity through processes such as deregulated redox homeostasis and DNA replication stress, and these changes may differ depending upon which oncogene is involved [128].

Ras-induced increases in ROS levels in immortalised cells have been suggested to lead to transformation, in response to DNA damage, instead of growth arrest [127]. Furthermore, oxidative-modified DNA may not be correctly sensed, or repaired [127]. Whereas, in normal cells, Ras-induced increases in ROS levels, accompanied by high oxidative stress, may lead to upregulation of p53 and p21, and ultimately senescence [127]. Overall, it has been suggested that oncogenic Ras causes ROS accumulation, and ROS scavenging plays a role in reducing cellular senescence [127].

How oncogenes such as Ras increase ROS has been investigated in a study which determined a model for oxidase function in cell transformation [129]. NADPH oxidases were the first systems identified to produce ROS in a targeted manner, not as a by-product [130]. It was therefore shown that Ras upregulated the expression of one of the NOX family of proteins, NOX1 [129]. Subsequently, it is the ROS

produced by NOX1 that plays a role in cell transformation and tumourigenicity by the Ras oncogene [129].

In later study, which compared the cellular responses of the oncogenes Ras and Myc, the DNA damage response was shown to be engaged early on in Myc-overexpressed cells, and the speed of fork progression quickly reduced [128]. Then, in Ras-overexpressed cells, DNA replication stress resulted in later engagement of the DNA damage response, taking longer to slow fork progression [128]. This study also showed that mitochondria are not the source of ROS detected by the oxidative damage marker, 8-oxoguanine, after Ras or Myc induction [128]. Therefore, a model has been proposed suggesting that oncogenes induce DNA replication stress, which may be associated with ROS-induced signalling or damage, but that occurs independently of mitochondrial ROS production [128].

Cancer stem cells have been suggested to survive radiation, while the remaining tumour is removed, through enhanced ROS defences and reduced ROS levels [131]. The increased expression of free radical scavenging systems may be involved in lowering ROS in some cancer stem cells [131]. In further consideration of how cancer cells may compensate for increased ROS, the role of NRF2, a transcription factor involved in the defence against oxidative stress, is contentious. In one study, oncogene-induced NRF2 activation has been implicated in promoting ROS detoxification and tumourigenesis [132]. Then, in another study, NRF2-deficiency was shown to play a role in metastasis [133]. Consequently, a model of human mesenchymal stem cell stepwise transformation was employed for ROS changes during tumourigenesis [134]. This study showed that NRF2 repression by

oncogenes acted as an adaptive response in some cancer cells for the acquisition of a pro-oxidant state, favouring cell survival and tumour growth [134].

As discussed, a controlled intracellular ROS level ultimately contributes to cell proliferation and survival. Another study has shown that cancer cell antioxidant responses are enhanced through inhibition of the glycolytic enzyme pyruvate kinase M2 by ROS [135]. Specifically, glucose flux was found to divert into the pentose phosphate pathway, to enable sufficient reducing potential for ROS detoxification [135]. This study therefore indicates a role for metabolic alterations in both proliferation, and protecting against oxidative stress [135].

3.2.5 Antioxidant repair pathways

3.2.5.1 NRF2 pathway

Cells are protected against free radical damage by enzymatic and non-enzymatic antioxidant systems, and these systems can work synergistically [136]. Antioxidants can be endogenous or obtained exogenously, in the diet or as dietary supplements [136]. Glutathione peroxidase, catalase and superoxide dismutase are said to be contained in the most efficient enzymatic antioxidants [136]. Non-enzymatic antioxidants include Vitamin E and C, and thiol antioxidants such as glutathione and thioredoxin [136].

The NRF2-antioxidant response element signalling pathway is a major mechanism involved in the defence against oxidative stress [137], and its pathway is summarised in Figure 5. Briefly, under basal conditions NRF2 activity is repressed through its cytoplasmic tethering and ubiquitination, mediated by the redox-sensitive

Kelch-like ECH-associated protein 1 (KEAP1) [138, 139]. Changes in local redox conditions will affect KEAP1 because it is cysteine-rich, and it is therefore sensitive to deviations from basal conditions [138, 139]. There are two proposed mechanisms by how NRF2 becomes activated by KEAP1, through the imposed conformational change mediated by the cysteine residues [138]. The “hinge and latch” model suggests that the KEAP1 interaction is disrupted with NRF2, and the second model suggests Cul3, which is usually involved in repressing NRF2 by promoting its ubiquitination and proteasomal degradation, is dissociated from KEAP1 [138, 140]. Subsequently, KEAP1 becomes inactivated so that newly synthesised NRF2 can bypass repression and translocate to the nucleus [138]. Once translocated, the transcription factor NRF2 binds to the antioxidant response element (ARE) in the regulatory regions of antioxidant target genes, such as NAD(P)H:quinone oxidoreductase 1 (*Nqo1*), to coordinate an adaptive response to oxidative stress [138]. One study has shown that NQO1 can act as a direct ROS scavenger, and it can also play an antioxidant role [141].

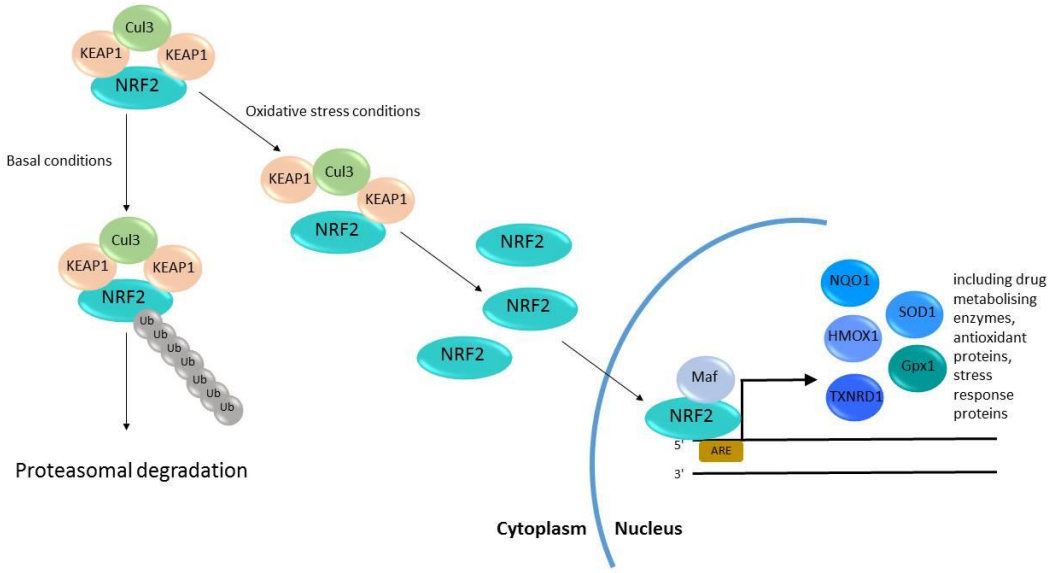


Figure 5. The NRF2 pathway.

Under basal conditions, the NRF2 protein undergoes proteasomal degradation, while under oxidative stress conditions, the NRF2 protein acts as a transcription factor for drug metabolising enzymes, antioxidant proteins and stress response proteins (adapted from: [138-140, 142]).

3.2.5.2 NQO1 plays a role in the oxidative stress response and cancer

NAD(P)H:quinone acceptor oxidoreductases are present as two major forms in mammalian systems, NQO1 and NQO2 [143]. Under normal and oxidative stress conditions, *Nqo1* gene expression is regulated by the antioxidant response element (ARE) [144].

NQO1 is a widely distributed FAD-dependent flavoprotein and it has greatly increased gene expression when there is stress on the cell, including oxidative stress and toxicity [145]. It also has increased gene expression in response to ionising radiation, heat shock, electrophiles, hypoxia, and heavy metals [146]. Quinone reduction and antioxidant activity, either indirectly through ubiquinone and

Vitamin E derivatives or directly through superoxide scavenging, are roles employed by NQO1 to protect cells against these stresses [141, 147].

Quinones are reduced to hydroquinones by the obligate two-electron reductase NQO1, where NADH or NADPH are used as reducing cofactors, stopping redox cycling of xenobiotics [147, 148]. Therefore, NQO1 acts as a detoxification and antioxidant enzyme to protect cells against toxicity and free radical generation from xenobiotic containing quinone moieties [147]. Through this mechanism, NQO1 diverts quinone electrophiles from participating in reactions that could lead to sulfhydryl depletion, as well as bypasses the unstable and highly reactive semiquinone intermediate [145, 147]. It is also possible for the hydroquinone products to be further metabolised to glucuronide and sulfate conjugates, allowing excretion to be facilitated [145].

It must also be noted that NQO1 can act as either a detoxification enzyme or an activating enzyme, and this can be based on how stable the hydroquinone is after reduction [148]. In the case of Menadione, this quinone can be reduced to a stable hydroquinone by NQO1, and subsequently conjugated and excreted, or it can form a semiquinone and give rise to ROS generation through one-electron reductases [148]. These one-electron reductases compete with the beneficial two-electron reduction by NQO1, for Menadione [148].

Up- or down-regulation of ARE-mediated gene expression is through a balance of NRF2 and another transcription factor, BACH1, inside the nucleus [144]. Overexpression of BACH1 was shown to repress *Nqo1* gene expression and induction in response to antioxidant treatment [144]. Furthermore, NRF2 and

BACH1 were shown to compete with each other to regulate ARE-mediated gene expression [144]. In response to antioxidant treatment, NRF2 and BACH1 have both been shown to translocate to the nucleus, where BACH1 accumulated more slowly than NRF2 [144]. This antioxidant-induced delayed accumulation of BACH1 could subsequently downregulate ARE-mediated gene expression, and could therefore allow antioxidant enzymes to return to normal levels [144].

High expression of NQO1 has been associated with poor prognosis in cancers, such as breast cancer and ovarian cancer [149, 150]. NQO1 bioactivatable drugs, such as the quinone known as β-lapachone, could be used to deliver tumour-selective DNA damage and cell death [151]. NQO1 catalyses the two-electron oxidoreduction of β-lapachone to generate an unstable hydroquinone, and this then spontaneously reacts in a two-step back-reaction with oxygen to regenerate the original compound [151]. Significantly, around 60 moles of NAD(P)H can be oxidised by NQO1-dependent futile redox cycling, and this can generate around 120 moles of ROS in about 2 minutes [151]. This can ultimately cause increased DNA lesions and hyperactivation of PARP [151]. Therefore, this study showed that a combination of β-lapachone and PARP inhibitors is synergistic at nontoxic doses of both drugs in NQO1-overexpressing cancers, including breast cancers [151].

3.3 Targeting NQO1 overexpression

3.3.1 Diminutol

An affinity chromatography experiment with Diminutol identified NQO1 as a possible target, when the role of NQO1 in microtubule polymerisation was also been

investigated in Xenopus egg extracts [152]. It was discovered that Diminutol is a competitive inhibitor of NQO1 with respect to NAD(P)H, with a Ki of 1.72μM [152].

As previously discussed, NQO1 catalyses the two-electron reduction of quinones to hydroquinones, using NAD(P)H as an electron donor, thus preventing other enzymes from catalysing a one-electron reduction and potential free radical production [147, 148, 152]. When this was tested in Xenopus egg extracts, Diminutol was not shown to increase free radical production when microtubules were depolymerised, therefore this effect was independent of oxidative stress [152].

Based on the limited investigations of Diminutol, this makes it an ideal compound to carry out further studies, and there is also potential for application as a therapy in the treatment of cancer patients.

3.3.2 Dicoumarol

Derived from Coumarin, the chemically named 3,3′-methylenebis[4-hydroxycoumarin], also known as Dicoumarol, is a natural anticoagulant drug that has been used extensively in research [153, 154]. Dicoumarol is considered to be a potent NQO1 inhibitor, and it has also been suggested to play a role in interfering with mitotic spindle microtubule function and therefore inhibiting cell proliferation [153, 154].

As an NQO1 inhibitor, Dicoumarol acts as a competitive inhibitor by competing with NAD(P)H for binding to NQO1 and thus preventing electron transfer to FAD [155,

156]. Its potency relative to other NQO1 inhibitors, such as ES936, is indicated by its Ki value of 1-10nM, compared to 450nM, respectively [155, 156].

Dicoumarol has been used to target NQO1 in cancer cells, and it has shown to be effective at sensitising different cancer cells to Cisplatin, Doxorubicin and Gemcitabine [157-159]. In urogenital cancer cell lines, cytotoxicity of Dicoumarol was dependent upon NQO1 expression status [157]. Then, when investigating the role of NQO1 in Dicoumarol-mediated modulation of Cisplatin cytotoxicity, NQO1-depleted cells did not show enhanced sensitivity to Cisplatin by Dicoumarol [157]. This result therefore suggested that NQO1 is required for Dicoumarol-mediated modulation of Cisplatin cytotoxicity [157].

3.3.3 Other Coumarin-based compounds

Despite Dicoumarol being recognised as the most commonly used NQO1 inhibitor, it is not without its limitations. This has been shown in a study of the MTT assay, where it is normally included in the assay, the authors concluded on a recommendation that it should not be added [160]. This is because cell lines showed different sensitivities to the compound, and this may skew cell viability data when evaluating cytotoxins [160]. In a study of ovarian cancer, Dicoumarol was found to inhibit an overexpressed protein pyruvate dehydrogenase kinase 1, and the authors also acknowledged other anticancer effects of Dicoumarol through NQO1 inhibition [161]. These studies therefore illustrate how Dicoumarol may effect cell viability data and how there are potentially multiple cellular targets in cancer cells, so the effects may not always be mediated by NQO1 inhibition [160, 161].

Based on these limitations, there was a subsequent need to achieve both greater potency and greater selectivity for inhibiting NQO1, than that of Dicoumarol [162]. To investigate this, two sets of novel NQO1 inhibitors that were analogues and hybrid compounds, were firstly identified [163, 164]. From this, further studies showed that these compounds could lower toxicity and have fewer off-target effects, in addition to increased potency [162, 164, 165]. When testing the chemotherapeutic agent EO9, which is normally activated by NQO1 to generate highly cytotoxic species, it was observed that some of their compounds were more efficient than Dicoumarol at inhibiting NQO1, and that they had greater protection against EO9 toxicity [162]. Additionally, the authors commented on Dicoumarol as being one of the most toxic compounds in all cell lines, and it is unclear whether factors such as NQO1 status play a role in determining the level of toxicity [165]. It was then suggested that the toxicity of Coumarin analogues may be related to superoxide generation, however exceptions would exist [165].

3.3.4 Irreversible inhibitors

Even the Coumarin analogues are not without limitations, and this is based on the competitive inhibition nature of these compounds, as with Dicoumarol [155, 156]. This property actually impedes their use in the evaluation of the activating or detoxifying properties of NQO1 [155, 156]. To overcome this, mechanism-based inhibitors such as ES936 have also been investigated [166]. In this instance, rather than identifying a potential therapeutic treatment option, this study has identified a compound that can be used as a tool to further define the role of NQO1 [166].

3.4 FOXM1 in cancer

3.4.1 Overexpression of FOXM1 in different cancer types

FOXM1 has been shown to be overexpressed in early and later stages of breast cancer development [167]. It has also been shown that FOXM1 expression correlated with HER2 status, suggesting FOXM1 may represent an important target in treating HER2-resistant breast cancer tumours [168]. In another study, this time in the oestrogen receptor-positive subtype of breast cancer, FOXM1 overexpression was found to be associated with poor disease free and overall survival [169]. Furthermore, Tamoxifen resistance had been linked to increased expression of FOXM1 and an increase in cancer stem cells, in oestrogen receptor-positive breast cancers [170]. In a very recent study of breast cancer, the overall assessment of FOXM1 and different breast cancer subtypes suggested that triple-negative breast cancer cells had the highest expression of FOXM1, compared to other subtypes [171].

Integrated genomic analyses of ovarian carcinoma were used to show pathway alterations in high-grade serous ovarian cancer, using clinically annotated samples [172]. Notably, the FOXM1 transcription factor network was found to be activated in 84% of cases [172]. As previously discussed, p53 represents a repressor of FOXM1 after DNA damage, so the high rate of p53 mutation in high-grade serous ovarian cancer could contribute to overexpression of FOXM1 [104, 172]. When the expression status of FOXM1 was investigated in ovarian cancer, it was observed that high FOXM1 expression correlated with high-grade tumours [173]. FOXM1 overexpression was found in 73% of Grade 3 tumours, while 68% of Grade 1 and Grade 2 tumours had low FOXM1 expression [173]. It has also been shown that overexpression of FOXM1 correlates with poor patient survival [174].

In a comparison of bladder tumour samples to normal tissue, FOXM1 was among a list of five genes that were identified to be overexpressed in tumour samples, which were involved in promoting cell proliferation and growth [175]. When invasively growing T24 bladder cancer cells and poorly invasive bladder cancer cells, BIU-87, were investigated it was determined that FOXM1 expression was higher in T24 bladder cancer cells [176]. When investigating FOXM1 with clinicopathologic parameters, of the 63 bladder cancer patients to experience recurrence, 60 also had high FOXM1 expression levels [176]. This was in contrast to the 37 patients who did not experience recurrence, and only 18 also had high FOXM1 expression levels [176]. In addition, FOXM1 was shown to be increased from low- to high-grade bladder cancers [177].

High levels of FOXM1 were observed in 85% of colorectal cancer tissue samples, compared to only 20% in adjacent non-cancerous tissue samples [178]. The colorectal cancer cell line LOVO was used as a model because of having high FOXM1 expression, and it was subsequently found that when FOXM1 was depleted in these cells this caused suppression of cell growth, invasion, and metastasis [178]. Therefore, FOXM1 may be involved in initiation and progression of colorectal cancer [178]. It was also found that poor patient survival was associated with high FOXM1 expression in colorectal cancer patients [179].

3.4.2 FOXM1 as a novel candidate for cancer therapy

FOXM1 was identified to be overexpressed in several types of solid tumours, including breast, ovarian, bladder, lung and colon [180]. A correlation of FOXM1 and human cancer was shown with high expression levels of FOXM1 in human basal cell carcinomas, and that this was a target of the glioma transcription factor-1,

Gli1 [181]. In a study of UVB and FOXM1 in skin cancer initiation, aberrant upregulation of FOXM1 was described as a “first hit” in cells, causing genomic instability [182]. UVB-activation of FOXM1B was shown to be dose-dependent [182]. The “second hit” has been suggested to occur when the DNA-damage checkpoint response ceases to exist, such as inactivation of p53, subsequently leading to cancer initiation [182]. In breast cancer, FOXM1 was identified as one of seven early molecular markers [167]. Furthermore, FOXM1 was found to be significantly overexpressed in ductal carcinoma *in situ*, and it was even more highly expressed in invasive ductal carcinoma [167]. In this instance, FOXM1 as a molecular marker may assist in earlier detection of breast cancer [167]. In continued consideration of other breast cancer studies, it was found that Thiostrepton could be used to selectively target FOXM1, and that cell proliferation and tumour growth are suppressed when FOXM1 is depleted, in breast cancer cells [183, 184]. Therefore, FOXM1 represents a molecular marker and a novel candidate for cancer therapy [167, 180, 183, 184].

Significantly, the previously discussed roles of FOXM1, such as regulating the DNA damage response and oxidative stress response, make targeting FOXM1 even more of an attractive therapeutic option [185]. In one investigation, it was observed that cancer cells were sensitised upon FOXM1 suppression, by RNA interference or FOXM1 inhibition, to DNA-damaging agent-induced apoptosis [186]. In another investigation, FOXM1 inhibition and ROS inducers in combination were shown to induce apoptosis in cancer cells [187]. Therefore, these studies show evidence that a combinatorial anticancer therapy may also be considered as an effective way to treat patients [186, 187].

3.4.3 FOXM1 and chemoresistance

As previously mentioned, the role of FOXM1 in regulating members of DNA repair pathways has been linked to chemoresistance. Briefly, RAD51 re-expression was shown to partially rescue Temozolomide resistance in FOXM1-depleted recurrent glioblastoma cells [116]. FOXM1- or BRIP1-depleted Epirubicin-resistant cells could be resensitised to Epirubicin [117]. In breast cancer cells treated with Epirubicin, overexpression of FOXM1 led to increased NBS1 expression and ATM phosphorylation, while in FOXM1-depleted breast cancer cells this resulted in reduced NBS1 expression and ATM phosphorylation [118]. As with FOXM1, NBS1 was also overexpressed in Epirubicin-resistant cells, and the parental cells showed low but Epirubicin-inducible levels [118].

In other studies of breast cancer, acquired resistance to Cisplatin has been suggested to be mediated by FOXM1 and enhanced DNA-damage repair pathways [188]. FOXM1 has also been shown to mediate resistance to Herceptin and Paclitaxel in breast cancer, and in this instance it is suggested to be through altered microtubule dynamics [189]. In both studies, targeting FOXM1 enabled cells to be resensitised to therapy, and this could subsequently be applied in other cancer types [188, 189]. Additionally, FOXM1 has been shown to mediate resistance to Paclitaxel in ovarian cancer, and KIF2C, a novel transcriptional target of FOXM1, may also be implicated in Paclitaxel resistance [174]. The roles of KIF2C include maintenance of spindle assembly during mitosis and meiosis, and this protein has already been shown to be involved in carcinogenesis [174]. Inhibition of FOXM1 has resulted in increased sensitivity to Cisplatin, Carboplatin, Doxorubicin Hydrochloride, and Olaparib, in ovarian cancer, again illustrating the role of FOXM1 in driving chemosensitvity [190].

FOXM1 and FOXM1 targets have also been considered in relation to evasion of apoptosis [191]. In this case, FOXM1 was shown to be a transcriptional activator of the anti-apoptotic genes XIAP and Survivin [191]. Furthermore, overexpression of FOXM1, XIAP, and Survivin has been suggested to contribute to the development of resistance to Docetaxel and Doxorubicin [191].

4.0 Targeting FOXM1 overexpression

It is widely discussed that transcription factors are deemed to be less “druggable” than, for example, enzymes [192, 193]. This is because when developing drugs that target transcription factors, they must be able to specifically disrupt or recruit DNA-protein or protein-protein interactions, to directly modulate protein function [192]. To overcome these limitations, several approaches have been investigated, including the use of screens to identify FOXM1 inhibitors [194].

4.1 Thiazole antibiotics and proteasome inhibitors

4.1.1 Siomycin A

In a cell-based screening system of compound libraries from the National Cancer Institute, the antibiotic thiazole Siomycin A was identified as a potent inhibitor of FOXM1 transcriptional activity [194]. It was also found that Siomycin A was able to inhibit anchorage-independent growth and induce apoptosis in transformed cells, whilst sparing normal cells [194]. In further mechanistic studies, it was shown that Siomycin A inhibits FOXM1 expression, but not the expression of other members of the Forkhead box family, indicating that this is a FOXM1-specific inhibitor [195].

Downregulation of FOXM1 with Siomycin A was shown to sensitise glioblastoma cells to irradiation, and these cells showed significantly increased DNA double-strand breaks as well as enhanced radiation induced mitotic catastrophe [196]. This may not be effective in all cancer types, as when this strategy was applied to radioresistant melanoma cells, these cells were not resensitised [197]. That said Siomycin A was proven to be effective as a single agent, inducing cell death in metastatic melanoma cells with constitutive FOXM1 expression [197].

4.1.2 Thiostrepton

Following the identification of Siomycin A, another thiazole antibiotic with small structural differences to Siomycin A was discovered to inhibit FOXM1, known as Thiostrepton [183, 198]. As described for the specificity of Siomycin A, treatment with Thiostrepton causes FOXM1 expression to be specifically inhibited in cells [198]. Therefore, not only do thiozole antibiotics downregulate transcriptional activity, but they also downregulate the expression of FOXM1 at the protein and mRNA levels [198]. This decrease in protein expression of FOXM1 that is detected after Thiostrepton treatment is suggested to be attributed to the positive auto-regulatory loop of FOXM1 [199].

In a study where Thiostrepton was identified as a potential anticancer agent, its selectivity for inducing cell death only in breast cancer cells and not in nontransformed breast epithelial cells was shown to be through downregulation of FOXM1 expression [183]. The authors also suggested that the reason for nontransformed cells not being sensitive to Thiostrepton is related to a tolerable toxicity of the compound, which may not be the case for cancer cells with much higher FOXM1 expression [183]. Furthermore, Thiostrepton was shown to inhibit

migration and invasion in addition to inhibiting tumour growth, in ovarian cancer cells [200]. Thus targeting signalling pathways which involve FOXM1, with Thiostrepton, could be used as a way of combating aberrant activation of such pathways in ovarian cancer [200]. Targeting FOXM1 with Thiostrepton has also been shown to induce apoptosis, as well as exhibit chemosensitising activity, in medulloblastoma cells [201]. When investigating the role of FOXM1 in 5-Flourouracil resistance, a synergistic effect was subsequently observed when FOXM1 was targeted in cells treated with Thiostrepton, in combination with 5-Flourouracil [202]. It was suggested that FOXM1 partially mediates resistance through regulating the main target of 5-Flourouracil, thymidylate synthase enzyme [202].

Interestingly, in an investigation of the thiopeptides Siomycin A and Thiostrepton, these compounds were reported to also act as proteasome inhibitors [195]. The stabilisation of proteins, such as p21, that are usually upregulated during proteasome inhibition, facilitated the discovery of the proteasome inhibitor activity of Siomycin A and Thiostrepton [195]. The authors investigated some well-known proteasome inhibitors, including Bortezomib, and these were also found to inhibit FOXM1 transcriptional activity and expression [195]. Therefore, this property of negative regulation of FOXM1 by proteasome inhibitors could be exploited for their anticancer properties [195].

When considering the mechanism of action of Thiostrepton, different concepts have been proposed in the literature [203, 204]. One suggested mechanism is that FOXM1 is bound directly by Thiostrepton [203]. The authors state that this interaction prevents the interaction of the transcription factor with several gene

promoters, but not necessarily to its own [203]. Another mechanism relates to Thiostrepton as an inhibitor of transcriptional activity and FOXM1 expression, via proteasome inhibition [204]. It suggests that proteasome inhibitors increase the expression of a putative negative regulator of FOXM1, and this binds to the promoter of FOXM1 to interrupt the autoregulation of transcription of FOXM1 [204]. Furthermore, this may explain how a number of proteasome inhibitors can block transcriptional activity and expression independently of their structure [204]. HSP70 was later identified as the negative regulator of FOXM1, after proteotoxic stress, through binding FOXM1 and interfering with its DNA binding action [205]. The authors also discussed how targeting HSP70 as a therapeutic strategy in cancer is becoming more prominent and that this should be developed in combination with FOXM1 inhibitors, because of their work showing that HSP70 inhibition leads to upregulation of FOXM1 [205].

4.1.3 Bortezomib

As previously discussed, the thizole antibiotics Siomycin A and Thiostrepton act as proteasome inhibitors, and that inhibition of FOXM1 by these compounds can also be achieved by well-known proteasome inhibitors, such as Bortezomib [195]. Notably, Bortezomib was the first proteasome inhibitor to enter clinical practice [206]. However, restrictions on Bortezomib use have been identified, and these include dose limiting toxicity [206]. For this reason, second generation proteasome inhibitors are now in clinical development [206].

One group investigated the potential for a combination therapy of Thiostrepton and Bortezomib, to allow for a decreased chance of drug resistance emergence, and to use subtoxic concentrations [207]. As thiopeptides have been shown to cause

suppressed FOXM1 expression and induction of apoptosis in cancer cells, it has been suggested, and subsequently shown, that Bortezomib could act synergistically with these compounds [198, 207, 208]. It was observed that Thiostrepton and Bortezomib in combination caused apoptosis and inhibition of long-term colony formation in different cancer cell lines, and that the relative level of FOXM1 may affect the sensitivity of cells to this combination treatment [207]. Importantly, when a modified form of Thiostrepton, without proteasome activity, was used in combination with Bortezomib, the synergy was lost, and therefore suggests that proteasome activity of these compounds is required for a synergistic action [207].

4.2 Small molecules and other FOXM1 inhibitors

Despite the previously discussed promising investigations of thiazole antibiotics and proteasome inhibitors in targeting FOXM1, these compounds are not without limitations, such as off-target effects [209]. To identify inhibitors of FOXM1 that block DNA binding, a high-throughput screening assay of just over 50,000 small molecules was carried out and novel inhibitors were discovered [209]. The lead inhibitor, NCGC00099374 (FDI-6), was further investigated and it was found to target FOXM1 and displace it from chromatin to reduce its transcriptional activity [209]. This study therefore offers the chance to explore other opportunities to target FOXM1 overexpression in cancer [209, 210].

In another high-throughput screening approach, the novel small-molecule compound RCM-1 was identified, and it was found to inhibit FOXM1 activity [211]. As opposed to inhibiting FOXM1 and its binding to its target DNA, FOXM1 targeting was through inhibition of its nuclear localisation [211]. Therefore, because of the auto-regulatory function of FOXM1 [199], the reduced *Foxm1* mRNA that was

observed in this study where mice had been treated with RCM-1, was seemingly as a consequence of the reduction in FOXM1 protein abundance caused by RCM-1 [211].

The small molecule Honokiol has been previously shown to be effective in cancer cells at suppressing Ras activation [212], and more recently it has also been shown to bind and inhibit FOXM1 [213]. The authors demonstrated that inhibition of FOXM1 with Honokiol induced apoptosis, and that structural analogues of this compound were not able to reproduce the same effect, therefore suggesting that there is a required structural specificity to inhibit FOXM1 [213].

Another FOXM1 inhibitor, known as an ARF peptide inhibitor, has shown to be effective at limiting proliferation and inducing apoptosis of liver cancer cells *in vivo* [214]. Despite the promising results of studies like this, it has also been discussed that there may be unwanted immunological responses to the peptide structure, associated with long-term administration of the peptide [215]. This could therefore render a decreased effectiveness of this treatment in cancer patients [215].

5.0 DNA damage response and oxidative stress, and their roles in cancer

5.1 DNA damage

5.1.1 Endogenous and exogenous DNA damage

Hydrolysis, exposure to reactive oxygen substances and other reactive metabolites are some of the chemical events that happen inside the cell that lead to DNA damage [216]. Reactive oxygen species (ROS) production from normal cellular metabolism is the major source of endogenous DNA damage, and this will be discussed in more detail in the next section [217]. Environmental agents that are known exogenous sources of DNA damage include ultraviolet light, ionising radiation, chemicals, toxins and pollutants [217]. Cancer treatments are also forms of exogenous DNA damage, such as chemotherapy and ionising radiation, and they exert their cytotoxic effects by inducing DNA damage through various mechanisms, including DNA double-strand breaks [218].

In the case of hydrolysis, this can occur in the form of DNA hydrolysis where the glycosidic bond between bases and deoxyribose can be labile under conditions such as alkylation of bases, and this can lead to an abasic site once cleaved [216]. Notably, abasic sites are estimated to occur at a rate of 10000 lesions/human cell/day, and these are considered to be one of the most frequent endogenous lesions [216]. If abasic sites are bypassed and subsequently left unrepaired, they may become mutagenic [216, 219].

Other examples of endogenous DNA damage include hydrolytic deamination of cytosine to generate uracil, an aberrant base [220]. To overcome this potential for mutations to occur, base excision repair may be initiated in the cell [220].

5.1.2 Reactive oxygen species and oxidative stress

ROS is endogenously and exogenously produced by sources such as oxidative metabolism in the mitochondria and ionising radiation, respectively [217]. In normal cells, ROS generation is highly regulated, where ROS are required as signalling molecules and for cellular homeostasis [221, 222]. To maintain a healthy status, equilibrium between oxidants and antioxidants must be achieved, and an imbalance is what leads to oxidative stress and consequently oxidative damage. Therefore, when generation of ROS becomes uncontrolled and exceeds levels of antioxidants, the result is oxidative stress and damage to lipids, proteins, and nucleic acids, because ROS will react with these macromolecules [217]. This can subsequently lead to the pathogenesis of age-related and chronic diseases, such as cancer [217].

Free radicals of ROS have unpaired electrons that are highly unstable and active towards chemical reactions with other molecules [223]. There is another group of ROS known as nonradicals, formed when two free radicals share their unpaired electrons [223]. The superoxide anion, hydroxyl radical, and hydrogen peroxide are three major ROS that are of physiological relevance [223]. Superoxide anion, for example, is formed by the addition of one electron to molecular oxygen [223]. Usually, electrons are transferred down the electron transport chain, however some electrons will leak and this is what allows superoxide to form [223].

Cancer cells have been said to be characterised by high levels of ROS and persistent oxidative stress, to further promote cancer growth and metastasis of the cell clones [217]. Increased ROS may lead to the activation of oncogenes and oncogenic signals, resulting in processes including cell proliferation and inactivation of tumour suppressor genes [222]. These conditions of oxidative stress have caused neoplastic cells to develop powerful antioxidant mechanisms [224]. Hence ROS represent a double-edged sword in cancer treatment, and whether clinically therapeutic benefits can be achieved through antioxidant supplementation or inhibition [222].

The oxidised DNA base, 8-hydroxy-2-deoxyguanosine, has been recognised as a potential marker of target organ damage from ROS, and it is one of the most abundant oxidative-modified lesions in DNA [225, 226]. 8-hydroxy-2-deoxyguanosine is a mutagenic lesion which can be mispaired with adenine, and during the next replication this wrongly paired adenine will cause a transversion by pairing with thymidine [226]. Its level correlates well with cancer incidence, and it has also been shown to be increased in breast tumours compared to noncancerous controls [225]. Another study has shown that there are significant differences between the control group and group with advanced disease, when investigating the involvement of oxidative stress during the progression of cervical carcinoma [226].

5.2 DNA repair pathways

5.2.1 Repairing DNA single-strand breaks

The DNA damage response is a collective term used to describe the mechanisms that cells have evolved to respond to and ultimately protect themselves against

DNA damage, so that they can detect, signal and repair any DNA lesions [227]. There are a number of DNA repair pathways that cells use to repair DNA lesions, and this can be for the repair of single- or double-strand breaks [227].

Single-strand breaks (SSBs) can be introduced into DNA through a variety of ways, including ROS and even erroneous activity of enzymes, such as DNA topoisomerase 1 where a transient nick in the DNA can sometimes become a lesion [228]. Importantly, unrepaired SSBs can block or collapse DNA replication forks and lead to double-strand breaks (DSBs), and whilst DSB-repair pathways exist in the cell, acute increases in cellular SSB levels may cause pathway saturation, leading to genetic instability and/or cell death [228].

As SSBs represent the most frequent form of genomic damage, PARP-1 plays a vital role as a stress sensor for this type of DNA lesion [229]. In a structural study of PARP-1, it was revealed that there is a tight spatio-temporal control of catalytic activity, as well as subtle degrees of regulation, alongside its rapid response to SSBs [229].

The mismatch repair (MMR) pathway is required for the removal of base-base mismatches that can give rise to point mutations and insertion/deletion loops leading to frameshift mutations. Briefly, MutSα/MutSβ recognises the mismatch and subsequent binding of MutLα/MutLβ to MutSα starts the process of mismatch excision which is followed by EXO1 recruitment to enable removal of nucleotides [230]. Once removed, DNA polymerase δ is involved in the DNA resynthesis step and DNA ligase seals the nick [230].

The base excision repair (BER) pathway is required for the removal of non-bulky base damages. Abasic sites are created in BER, and these are recognised by endonuclease 1 so that the DNA backbone is then cleaved to allow for repair to be initiated by DNA polymerase β [230].

The nucleotide excision repair (NER) pathway is for the removal of bulky DNA lesions. The XPA protein and the XPC–HR23B complex are suggested to detect a lesion in NER [231]. The next steps involve DNA melting and opening of the repair section by the XPB and XPD helicases of TFIIH, and then the XPF and XPG endonucleases excise the damaged DNA so that the re-synthesise step can be carried out by the DNA polymerases α and δ [231]. These pathways all involve the repair of SSBs, and they also have SSB transients during the processing of DNA damage [230-232].

5.2.1.1 Role of PARP

Poly(ADP-ribose) polymerase (PARP-1) is an abundant nuclear protein, belonging to a family of at least 17 members [233]. Within the family, PARP-1 and PARP-2 are known to play a role in DNA repair, and they are localised to the nucleus [233]. PARP family members are also known to have other functions in the cell, including gene regulation, chromatin remodelling, and apoptosis [234]. All PARP proteins have a conserved catalytic domain, as well as other domains, such as zinc finger, BRCT, SAM, SAP, and ankyrin [235].

A nomenclature system has been used to classify PARP proteins, according to their motifs and functions: (1) PARP 1-5, have a conserved glutamate residue that

defines PARP catalytic activity; (2) PARP 6-8, 10-12, and 14-16, are putative mono-(ADP-ribose) polymerases; and, (3) PARP 9 and 13 which do not have PARP signature motif that binds NAD+ nor do they have the catalytic glutamate, implying that they are inactive [236].

The PARP-2 catalytic domain has the strongest resemblance to that of PARP-1 with 69% similarity [237]. PARP-3 has been identified as a core component of the centrosome [237]. PARP-4 is the largest member of the family, and it was discovered associated with vault particles [237]. The function of these particles is unknown; however, they could be associated with multidrug resistance in some cell lines [237]. Tankyrase 1 (TRF1-interacting, ankyrin-related ADP-ribose polymerase), (PARP-5a), which is involved in telomere maintenance, contains the smallest domain homologous to PARP-1 that still displays PARP enzymatic activity [237]. Furthermore, a second tankyrase (PARP-5b or tankyrase-2) encoded by a distinct gene has also been discovered [237]. The catalytic residue E988 of PARP-1 is conserved among PARP-1s from various species, in PARP-2, PARP-3, PARP-4 and in Tankyrases [237]. This Glu residue is also conserved in other members: PARP-5c, PARP-11, PARP-12 and PARP-14 [237].

There has been evidence suggesting a role for PARP-3 in the cellular response to DSBs, potentially acting with PARP-1 [238]. This study also found that PARP-3 may be functioning in a network linked to mitosis, with the mitotic factor NuMA and Tankyrase 1, linked to spindle microtubule dynamics and telomere integrity [238]. Furthermore, other studies have shown that PARP family members contain a group of IFN-inducible inhibitors of virus replication [239]. Specifically, PARP-7, PARP-10,

and the long isoform of PARP-12 have been demonstrated to be potent regulators of cellular translation and virus replication [239].

The catalytic ability of PARP-1 allows long polymers of ADP-ribose to be produced on it, and other proteins, by a process known as PARylation [240]. This polymerisation is stimulated by the binding of PARP-1 to a single-strand break in the DNA, and PARP-1 has been found to be required for the recruitment of essential factors in single-strand break repair, including the molecular scaffold protein XRCC1 [241]. This study determined that mutation of the XRCC1 BRCT I domain, which interacts with poly (ADP-ribose) polymer, reduced or prevented the appearance of XRCC1 foci [241]. Therefore, PARP-1 is indicated to be required for XRCC1 recruitment to single-strand break sites [241].

PARylation is a transient, reversible modification, and it is counteracted by the activity of PARglycohydrolase (PARG), which degrades PAR [242]. This study showed, for the first time, that PARG is critical for rapid rates of chromosomal SSB repair [242]. Then, PARP-1 and PARG deficiency together did not slow SSB repair any more effectively than reduction of either protein alone, such that an increased rate of SSB repair may be due to the proteins acting together [242]. The authors also suggested that the accumulation of XRCC1 protein scaffold at sites of damage may be regulated [242]. A later study subsequently showed a dynamic regulation of XRCC1, in response to damage, such that XRCC1 dissociates from PAR, and locates to sites of SSBs dependent on its BRCTII domain and PARG function [243]. The authors suggest a model where XRCC1 is recruited to PAR at the damage site through its BRCT I domain, while XRCC1 is kept at the site once PARG degrades PAR through its BRCT II domain [243].

PARP-1 has been suggested to essential to efficient base excision repair, where efficiency was reduced in PARP1-deficient cells [244]. In a study where PARP-2 was characterised, it was discovered that the PARP-1 and PARP-2 proteins homo- and hetero-dimerise and poly(ADP-ribosyl)ate each other [245]. Furthermore, PARP-2 was shown to interact with the base excision repair proteins XRCC1, DNA polβ, and DNA ligase III, which are all PARP-1 partners [245]. The authors noted that despite a low capacity to synthesise ADP-ribose polymers, PARP-2 is also an active player in base excision repair [245].

Another study has proposed that PARP inhibition causes PARP-1 trapping on the single-strand break intermediate formed during base excision repair, rather than there being an immediate role for PARP-1 in base excision repair [246]. The authors suggested that PARP-1 may have a role in recognising single-strand breaks, or other DNA ends in the DNA, and to increase the repair [246]. A later study then suggested that PARP-1 is involved in base excision repair of a subset of lesions, such that PARP-dependent and –independent pathways exist in the cell [247]. The authors described a role for XRCC1 and PARP-1 in the repair of single-strand breaks and purine lesions, whilst PARP-1 activity was suggested to not be required for the repair of pyrimidine lesions [247].

Other functions of PARP-1 in the DNA damage response include its suggested role in protecting the homologous recombination pathway from interference with the non-homologous end-joining pathway protein, Ku70 [248]. Therefore, PARP-1 could be playing a role in regulating the balance between these two repair pathways [248]. A later study consistent with these findings, that PARP-1 and Ku compete with each other to recognise double-strand breaks, showed how these proteins may

be acting at the damage site [249]. PARP-1 and the Ku complex are suggested to compete with each other for the double-strand break repair because, once PARP-1 has been loaded to the double-strand break, the Ku complex was removed via PARylation [249].

To maintain accurate replication and genomic stability, it is important that stalled replication forks become reactivated, and PARP-1 has been shown to play an active role in replication restart [250]. Mechanistic investigations found that PARP-1 is involved in the recruitment of MRE11; promoting resection of the DNA and restart replication forks [250]. Therefore, PARP-1 is suggested to have a role in the detection and repair of single-strand breaks [241], repair pathway regulation [248, 249], as well as detecting stalled or collapsed replication forks and replication restart [250].

5.2.2 Repairing DNA double-strand breaks

There are several ways that DSBs arise in the DNA, such as replication across a nick, giving rise to chromatid breaks during S-phase, ideally repaired by HR using the nearby sister chromatid [251]. Other DSBs caused by ROS or ionising radiation, for example, are often repaired by non-homologous end joining (NHEJ) because they usually occur when there is no nearby homology donor, or because they occur outside of S-phase [251]. SSBs are generated first, and when two closely spaced lesions of this type on anti-parallel strands occur, this is what causes a DSB [251]. Furthermore, a physical dissociation of the two DNA ends that are generated by a DSB can make repair difficult, and this can also cause inappropriate recombination with other genomic sites [252]. Generation of DSBs can induce mutations and

chromosomal translocations, and such genome changes may have tumourigenic potential [252].

5.2.2.1 Double-strand break repair and pathway choice

There are a number of ways the cell can repair a DNA double-strand break. The major conservative repair pathways are non-homologous end-joining and homologous recombination [253]. Whilst other competing repair pathways, including single-strand annealing and alternative end-joining also exist, for when free DNA ends arise in the cell [253]. The competing pathways often take place opportunistically, where vulnerable repair intermediates may divert along one of those pathways [253].

In non-homologous end-joining, the core machinery is composed of DNA-dependent protein kinase (DNA-PK) complex and ligase IV/XRCC4 [254]. The DNA end-binding Ku70/80 heterodimer and the DNA-PK catalytic subunit (DNA-PK_{CS}) are involved in phosphorylation events after a double-strand break has been induced [254]. DNA-PK_{CS} autophosphorylation enable DNA ends to be accessible for processing factors and DNA ligase, where ligase IV/XRCC4 is responsible for joining DNA ends [254]. Ku70/80 and XRCC4 have been proposed to interact, where XRCC4 may serve as a flexible tether between Ku70/80 and ligase IV [254].

The steps involved in HR are illustrated in Figure 6. Briefly, once ATM and ATR kinases have recognised the DSB, downstream proteins are then phosphorylated which include CHEK2, P53, BRCA1 and H2AX, and it is BRCA1, along with BARD1 and BRIP1, that provide a scaffold for other proteins to be organised at the repair site [255]. MRE11, RAD50 and NBS1 form the MRN complex which is involved in

DNA resection, and then BRCA2 is recruited to load RAD51 onto the DNA, assisted by RAD51B, RAD51C and RAD51D [255]. Strand invasion is then initiated by the RAD51 nucleoprotein filament, where the sister chromatid is used as a template to enable error-free repair to take place [255]. Therefore, RAD51 forms the central enzymatic component of HR.

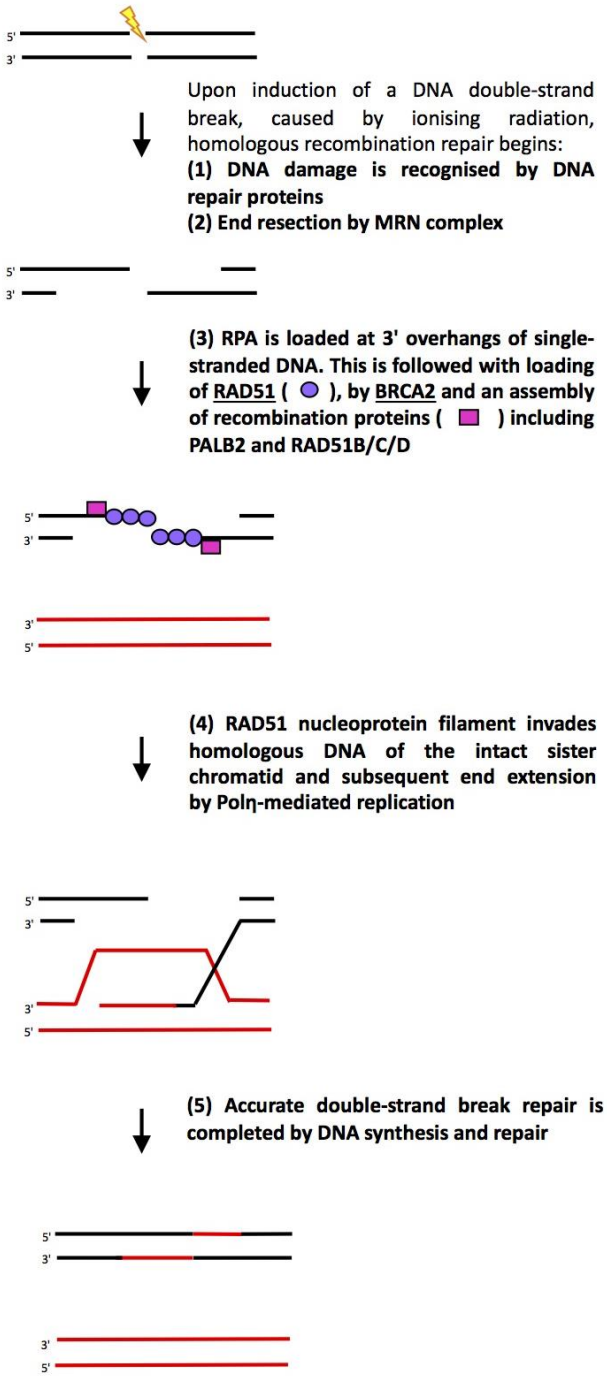


Figure 6. The homologous recombination pathway.

ATM and ATR kinases involved in step (1) recognise and initiate homologous recombination, and facilitate downstream phosphorylation of proteins such as CHK2, p53, BRCA1 and H2AX. BRCA1 scaffolds and organises DNA repair proteins like BARD1 and BRIP1, involved at step (1). The MRN complex which plays a role in DNA end resection in step (2) comprises of Mre11, RAD50 and NBS1. Then, it is during the unwinding of the double-stranded DNA that the proteins RPA, EXO1 and BLM can access, cleave and remove damaged sections, as shown in step (3). As illustrated, the central enzymatic protein, RAD51, can then be loaded onto the DNA by BRCA2. Finally, RAD51 nucleoprotein filament invades

homologous DNA in step (4) and accurate repair is completed in step (5). (Adapted from: [255-257]).

Single-strand annealing, another type of double-strand break repair pathway, involves annealing between DNA repeats that become single-stranded following resection of broken DNA ends, leading to deletions or changes to DNA structure [258]. This pathway is dependent on the key end resection factor, CtIP, and after end resection there is annealing of the flanking repeats, followed by removal of the non-homologous 3′ single-strand DNA tails, which are mediated by RAD52 and ERCC1 [259]. Gaps are then filled by DNA polymerases to allow DNA ligase to complete single-strand annealing [259]. Single-strand annealing may be important to restore a broken chromosome with double-strand break ends that have undergone extensive end resection, but are unable to be resolved other pathways, such as alternative end-joining pathway [259].

The alternative end-joining pathway is also initiated by end resection [259]. It is similar to single-strand annealing in that it involves annealing of flanking repeats to bridge the double-strand break [259]. While RAD52 plays a conserved mediator role in single-strand annealing, it appears dispensable for alternative end-joining [259]. Then, alternative end-joining seems to specifically require PARP and DNA polymerase theta [259].

Despite the mutagenicity of non-homologous end-joining, by blunt end ligation independently of sequence homology, its fast kinetics has a clear role in protecting genome integrity, particularly by Ku80 in the suppression of chromosomal translocations [260]. Then, if NHEJ cannot be completed, there are other pathways including homologous recombination, single-strand annealing, and alternative end-

joining, that can be used when DNA has been resected, leaving 3′ single-stranded DNA overhangs, demonstrating that the choice of repair mechanism is regulated throughout the cell cycle [261]. NHEJ is dominant in G1 and G2, even when both repair pathways are functional, and the highest proportion of breaks repaired by HR is mid-S-phase [261]. Furthermore, the absence of a sister chromatid for HR in G1 may support the use of alternative end-joining and single-strand annealing to repair resected double-strand breaks in this phase [262].

Further to the previously discussed PARP family members, PARP-3 has been suggested to limit end resection subsequently helping to decide between HR and end-joining pathways, partly in cooperation with the Ku70–Ku80 heterodimer [263]. This study found that PARP-3 PARsylates Ku70–Ku80, when recruited to DNA damage sites, caused limited DNA end resection during HR, while stimulated NHEJ. Then, when PARP-3 was deficient, they showed nevertheless that HR is significantly reduced, while enhanced end resection causes mutagenic deletions during alternative non-homologous end-joining [263].

HR and alternative non-homologous end joining both occur mostly in S- and G2-phases, and the mechanism of choice between these pathways has been investigated in a study linked to the roles of Polθ and RPA during DSB repair [264]. The authors suggest Polθ–helicase helps with the removal of RPA from resected DSBs to allow their annealing and subsequent polymerase joining by alternative non-homologous end-joining, making Polθ a potential novel cancer drug target [264].

5.2.3 RAD51 plays a role in DNA repair and cancer

Upon exposure to a variety of DNA-damaging treatments, subnuclear foci containing RAD51 form, and other recombination proteins including RAD52, BRCA1 and BRCA2, co-localise with RAD51 [265]. In fact, formation of these foci after DNA damage is dependent upon BRCA2 and RAD51 paralogues, indicating an ordered assembly is required, and to enable subsequent repair of damage [265]. RAD51 foci also appear during S-phase, and these are required to initiate stalled or broken DNA replication forks [266]. BRCA2 and RAD51C have both been implicated in facilitating nuclear transport of RAD51 [267].

Alongside its role in regulating nuclear transport and intracellular localisation of RAD51, BRCA2 has also been shown to regulate the DNA binding ability of RAD51 [268]. So, in cells that have BRCA2 inactivation, impairment in these controls may be involved in causing genomic instability, and tumourigenesis [268]. This study showed that the peptides corresponding to the BRC3 or BRC4 repeats of BRCA2 bind RAD51 protein and inactivate it with respect to its ability to bind DNA [268]. It was suggested that this inactivation was through an inability for RAD51 to self-associate, and therefore prevent nucleofilament formation, when there is no DNA damage [268].

Furthermore, when there is DNA damage, this interaction may also be involved in sequestering RAD51 in a ready-state to be brought to damaged sites, upon activation of the complex, to then play its role in DNA repair [268]. In *Brca2*-mutant cells, RAD51 may bind damaged DNA in a less productive manner, which may also affect RAD51's state of readiness as well as proper assembly of repair proteins at the damaged site, thus causing inefficient HR and genomic instability [268].

Importantly, increased nuclear RAD51 foci number and size represent a hallmark for early response to genomic damage [267]. Overexpression of RAD51 also increases nuclear RAD51 foci and then formation of higher order RAD51-chromatin complexes in the absence of DNA damage, and this has been shown to correlate with elevated HR, genomic instability and even increased radiation and drug-treatment resistance in cancer cells [267]. Hence nuclear RAD51 availability requires careful regulation to allow for the right amount of recombination, before and after DNA damage [267].

The role of RAD51 in breast cancer tumourigenesis has not consistently shown the same effects, such that in sporadic breast cancers the absence of the tumour suppressor protein BRCA1 and overexpression of wild-type RAD51 has been shown to contribute to pathogenesis, whilst another report showed reduced RAD51 in analysis of breast carcinomas [269, 270].

The functional observations from a study of pancreatic cancer indicated that RAD51 overexpression enhanced survival of cells after induction of DNA double strand breaks [271]. Furthermore, it was suggested that changes in RAD51 may contribute to the malignant phenotype for pancreatic cancer [271]. In another study, RAD51 overexpression was also identified in aggressive prostate cancer [266]. Therefore, elevated expression of RAD51 might have diagnostic value in identifying patients requiring more immediate treatment, and could be useful when analysing the biological basis of aggressive prostate cancer [266].

6.0 Synthetic lethality

The term synthetic lethality can be described as a type of genetic interaction in which occurrence of a specific genetic effect alone does not influence cell survival, whilst the co-occurrence of two specific genetic events results in cell death [272]. It is probably best known in the context of loss-of-function mutants, but other combinations of perturbations also cause synthetic lethality, such as the action of chemical compounds [272]. Therefore, the opportunity to exploit this mechanism offers the potential for the identification of novel cancer therapies [272].

6.1 Identification of synthetic lethal interactions using screening approaches

Compared to standard agents, synthetic lethal targeting of cancer cells is therapeutically advantageous in that this approach selectively kills cancer cells with a specific genetic mutation [273]. It also offers an increased therapeutic index, because of its selectivity for tumour cells, and therefore sparing of normal cells [273]. Another advantage of synthetic lethality in cancer treatment is that there is an opportunity to target loss-of-function mutations in tumour suppressor genes, which may have been previously more difficult to modulate with other strategies, because unlike oncogenes there is often no protein to inhibit [273].

Synthetic lethal interactions can be identified using screening approaches. In a genome-wide RNAi screen which investigated synthetic lethal interactions with the frequently mutated Ras gene in cancer cells, a number of genes were found, and subsequent inhibition of these proteins was shown to impair Ras mutant cell viability [274]. Not only does the screen offer therapeutic options, it also accounts for the

gene network supporting the RAS oncogene which can provide further insight into tumourigenesis [274]. In another study, genetic and chemical screening were integrated to identify E-cadherin synthetic lethal interactions for breast cancer patients, as these patients frequently have defects in E-cadherin, a tumour suppressor cell adhesion glycoprotein [275]. A drug-sensitivity screen, with drugs for clinical use for the treatment of cancer or in late-stage clinical development, and a parallel siRNA sensitivity screen were used, and a ROS1 receptor tyrosine kinase/E-cadherin synthetic lethal effect was discovered [275]. The synthetic lethality observed with ROS1 siRNA was statistically significant and ROS1 inhibitors, Foretinib and Crizotinib, were identified as candidate synthetic lethal drugs, suggesting that the ROS1 effect might be clinically tractable [275].

The implications of such approaches give rise to advantages and disadvantages, regarding therapeutic potential and translational benefits. As such, the advantages of RNAi screening include the ability to target any gene within the genome, whilst disadvantages include an inability to get a complete gene knockdown [276]. Then the advantages of drug screening include easier translation into clinical practice, whilst disadvantages include variability in drug specificity [276].

6.2 PARP inhibitors and beyond

Individuals who have with deleterious heterozygous germline mutations in *Brca1*/*2* tumour suppressor genes have significantly increased risks of developing breast, ovarian and other cancers, because this mutation can cause reduced function and less effective DNA repair mechanisms, which would normally act to prevent cancer [277]. Loss of the remaining wild-type BRCA allele through a somatic loss-of-function aberration occurs during tumourigenesis [277]. In HR-deficient cells, other

DNA repair pathways can become more predominant, and this can cause alterations to the DNA and could contribute to cancer initiation and progression [277]. Thus, HR-deficient cells require PARP activity, and PARP inhibitors offer a tumour-selective approach in cancer treatment [278] (Figure 7).

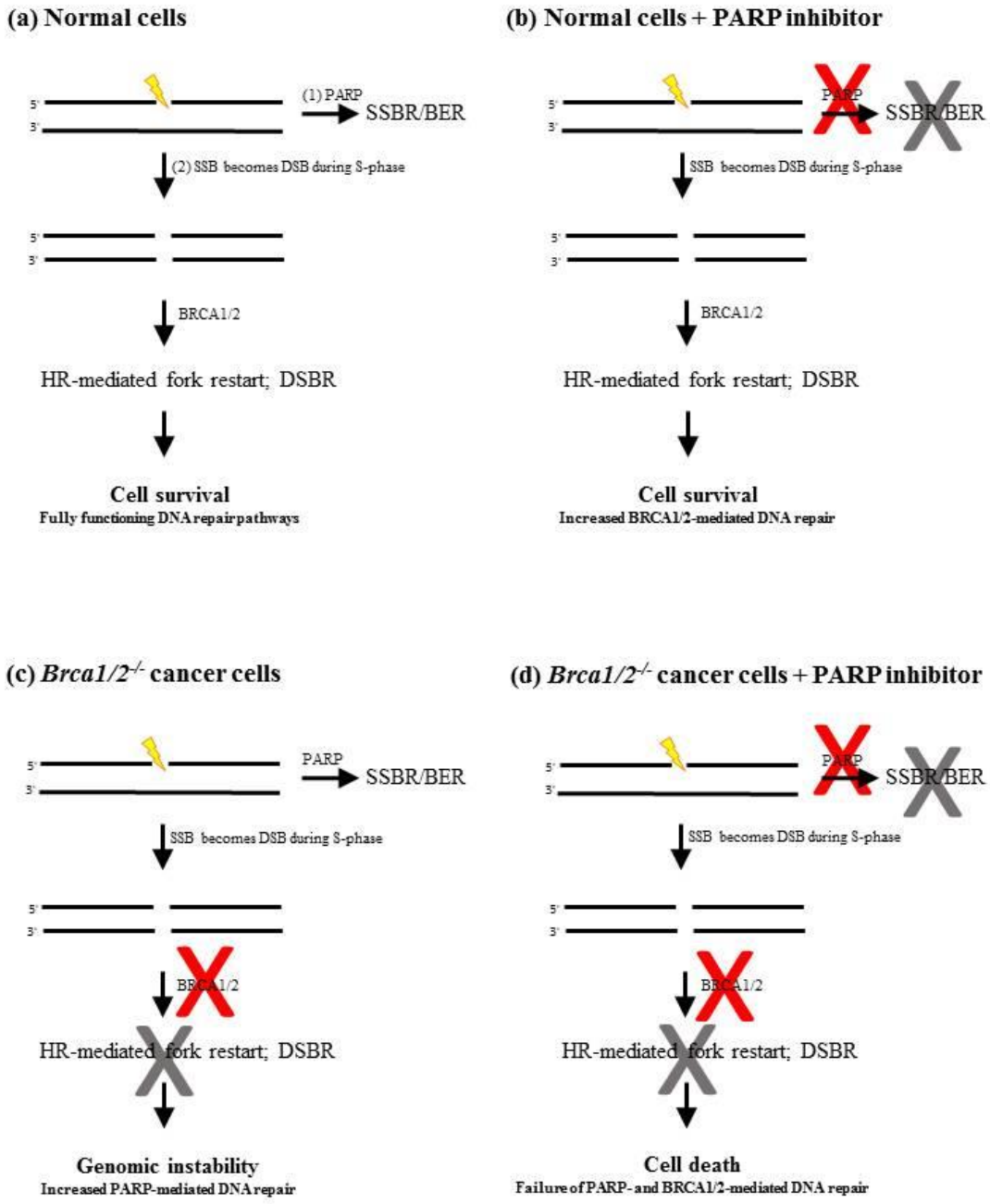


Figure 7. Synthetic lethality occurs in Brca1/2-deficient cells treated with a PARP inhibitor.

Normal cells (a) have fully functioning DNA repair pathways. When normal cells are treated with a PARP inhibitor (b), causing inefficient single-strand break repair, these cells are not susceptible to cell death because the DNA double-strand break repair pathways of these cells remain intact. While, in *Brca1/2*-deficient cancer cells (c), these cells have inefficient double-strand break repair mechanisms, and therefore have genomic instability. When *Brca1/2*-deficient cancer cells are treated with PARP inhibitor (d), causing inefficient single-strand break repair, these cells are susceptible to cell death because DNA double-strand break repair pathways of these cells are not intact. (Adapted from: [279, 280]).

The first synthetic lethal therapy has been approved for clinical use, and these include Olaparib and Rucaparib, which are collectively known as PARP inhibitors [40, 278, 281]. These are for patients with *Brca1*-mutant or *Brca2*-mutant ovarian and breast cancers, and the concept has been illustrated in Figure 7 [39, 40, 278]. An increase in DSBs or collapsed replication forks, initiated by PARP inhibition or PARP trapping, is said to be the cause of the synthetic lethal interaction between harbouring loss of BRCA function and PARP inhibition [277].

Olaparib represents the first PARP inhibitor to become available in clinical practice, where the greatest clinical benefit of this compound has been demonstrated for *Brca*-mutated patients, in clinical trials of patients with ovarian cancer [282]. It has also been considered to be well-tolerated by patients, where reported adverse effects have included fatigue and anaemia [282]. Furthermore, in proof of concept trials, *Brca*-mutated advanced ovarian cancer and *Brca*-deficient breast cancers showed favourable therapeutic index when treated with Olaparib [283, 284]. Response to PARP inhibition has also been shown to correlate with platinum sensitivity in ovarian cancer patients, however patients with platinum-resistant disease still had potential benefit, but these patients had a lower response rate [285]. In breast cancer patients, it was those who had HER2-negative metastatic breast cancer and a germline *Brca* mutation, where Olaparib monotherapy was shown to have a significant benefit over standard therapy [39].

In other studies, a tolerated dose of Olaparib in combination with Cisplatin was identified for patients with advanced solid tumours [286]. Antitumour activity was observed, and this was more evident in patients with germline *Brca1*/*2* mutations [286]. Furthermore, Olaparib in combination with Paclitaxel and Carboplatin,

followed by Olaparib maintenance monotherapy, was investigated in recurrent platinum-sensitive ovarian cancer patients [287]. This was shown to significantly improve progression-free survival, when compared to Paclitaxel with Carboplatin alone [287]. The greatest benefit was again for *Brca*-mutated patients, and there was an acceptable and manageable tolerability profile [287].

Furthermore, whilst the role of PARP inhibitors in relapsed ovarian cancer has been well-established, less was known about Olaparib as a maintenance therapy in newly diagnosed ovarian cancer, and so a trial was conducted to investigate this in these patients [288]. The findings of this study showed that Olaparib maintenance therapy benefitted patients with newly diagnosed advanced ovarian cancer and a *Brca1*/*2* mutation, for progression-free survival, and it was also shown that there was a 70% lower risk of disease progression or death with Olaparib compared to the placebo [288].

A catalytic mechanism of PARP inhibition has been proposed, as well as a PARP1-trapping model [289]. Since PARP inhibition was shown to delay SSB repair more than PARP deletion, this indicated that catalytic inhibition would not be the only mechanism of action of PARP inhibitors [289]. PARP1-trapping by PARP inhibitors is explained by PARP1 being trapped on DNA, forming PARP1-DNA complexes that interfere with DNA replication, and differing potencies have been described for different PARP inhibitors [289]. These potency differences may be important when designing combination therapies that include PARP inhibitors, to not exceed toxicity levels with other therapies such as chemotherapy when given in combination [277].

Rucaparib is another FDA-approved PARP inhibitor, and offers a new therapeutic option for patients with ovarian cancer [281]. As illustrated when Rucaparib was used in a trial of recurrent ovarian cancer patients following platinum therapy response, whilst maintenance therapies do offer improved progression-free survival, these effects may be transient so there is always a need to develop new therapies and optimise treatment strategies [290]. In this study, Rucaparib maintenance treatment improved progression-free survival compared with the placebo [290]. As observed in patients receiving Olaparib treatment, adverse effects of Rucaparib included fatigue [290].

Further studies of Rucaparib and ovarian cancer have suggested that quantitative BRCA1 methylation analysis could provide insight into PARP inhibitor response [291]. Near to the BRCA1 transcription start site, methylation of CpG sites is associated with reduced BRCA1 mRNA and protein, and when combined with loss of heterozygosity in the other allele, loss of functional BRCA1 results [291]. Methylation loss has been shown to occur after exposure to chemotherapy and can therefore be used as a predictor of response to Rucaparib, as well as emphasising that earlier introduction of PARP inhibitor therapy may also benefit patients [291].

Talazoparib is another FDA-approved PARP inhibitor, and can be used to treat patients with deleterious or suspected deleterious germline *Brca*-mutated, HER2-negative, locally advanced or metastatic breast cancer [292]. As with the other PARP inhibitors, there are adverse effects association with Talazoparib, and the most frequent of these is anaemia [292]. In a study of advanced disease breast cancer patients with a germline *Brca1/2* mutation, the outcome showed that

progression-free survival was favoured after single-agent Talazoparib, compared to standard chemotherapy [293].

As previously discussed, PARP inhibitors exert their mechanism by catalytic inhibition and PARP1-trapping [289]. Furthermore, it has been suggested that PARP1-trapping is more effective at causing cancer-cell death, rather than catalytic inhibition alone [293]. Interestingly, studies have shown Talazoparib to be a potent PARP inhibitor, not only because of its strong catalytic inhibition, but it also exhibits a PARP-trapping potential that is around 100 times greater than other PARP inhibitors under current investigation [293].

In one study, the DNA DSB response proteins ATM and DNA-dependent protein kinase (DNA-PK) were knocked out or inhibited to determine the role of these proteins in cellular survival, following PARP inhibition [294]. ATM was found to be required for PARP inhibitor-induced HR repair, where ATM was shown to be activated upon PARP inhibition [294]. Inhibition of DNA-PK was shown to make no difference to PARP inhibitor-induced HR, suggesting that PARP inhibition causes ATM to have additional functions in the pathway but not DNA-PK [294].

Use of PARP inhibitors may also be beneficial to more patients characterised by other mutations that lead to HR defects, rather than *Brca* mutations alone [295]. Patients with mutations in the tumour suppressor gene *PTEN* could be responsive to PARP inhibitors, where a study has shown synthetic lethal targeting of *PTEN* mutant cells with PARP inhibitors [295]. This is because besides its role as a phosphatase in the control of the phosphoinositide 3 kinase signalling pathway, PTEN has also been shown to have a role in genome stability, and PTEN-deficiency

gives rise to a HR defect which was shown to sensitise cells to PARP inhibitors [295].

Another study has shown a functional link between androgen receptor signalling and HR in prostate cancer [296]. The study showed that androgen receptor signalling regulates HR and promotes MRN foci formation, leading to ATM activation [296]. Therefore, inhibition of androgen receptor function is synthetically lethal with PARP inhibition [296].

Studies have also indicated how patient resistance can develop to PARP inhibitors, such as secondary mutations in *Brca2* [297, 298]. It was shown that resistance to therapy could be caused by intragenic deletion in BRCA2 [297]. By understanding the implications of these observations, this could not only provide insight into the mechanisms of drug resistance in *Brca* mutation carriers, it may also help in defining functionally important domains in BRCA2 [297]. Therefore, a screening approach could be adopted to assess for secondary mutations and response to PARP inhibitors, and therefore support the guidance of treatment decisions for patients [298].

Beyond PARP inhibition and BRCA deficiency, other DNA repair deficiencies have been exploited using a synthetic lethal approach. Tumour cells with the defective MMR gene *MSH2*, a tumour suppressor which forms the MutSα complex with MSH6, were shown to be selectively sensitive to Methotrexate [299]. In this study, a drug screen identified Methotrexate, which caused oxidative DNA damage in all cells [299]. This damage was repaired in *MSH2*-proficient cells but persisted in *MSH2*-deficient cells, indicative of the selectivity offered by this synthetic lethal

approach [299]. In a later study, tumour cells defective in *MSH2* or another MMR gene *MLH1*, a tumour suppressor which forms the MutLα complex with PMS2, were shown to be selectively sensitive to inhibition of particular DNA polymerases [300]. It was suggested that accumulation of the DNA base lesion 8-oxoguanine may have been responsible for the formation of lethal DNA breaks [300]. Another novel candidate therapeutic target for cancers deficient in the MMR pathway was identified, using parallel siRNA screens with tumour cell models either proficient or deficient in *MSH2* or *MLH1* [301]. The results showed that silencing of PTEN-induced putative kinase 1 (*PINK1*) was synthetically lethal in cells with *MSH2*, *MLH1*, or *MSH6* dysfunction [301].

Aims

The transcription factor Forkhead Box Protein M1 (FOXM1) has been identified to be overexpressed in several types of solid tumours, including breast, ovarian, bladder, lung and colon [180]. Therefore, FOXM1 represents an attractive therapeutic target in the treatment of cancer patients. However, a number of studies have also implicated FOXM1 in chemoresistance, suggesting that targeting FOXM1 alone may not translate into the clinic as a potential treatment strategy [116-118, 174, 190]. Our aim was to investigate novel combination therapy strategies, using a two-pronged approach, by targeting FOXM1 and exploiting the role of FOXM1 in regulating DNA repair genes and oxidative stress genes as a second target [116-119, 302, 303]. To this end, we aimed to:

Investigate Thiostrepton and Olaparib as a combination therapy and its mechanistic implications

Investigate the role of FOXM1 in the oxidative stress response and its therapeutic implications

Materials and Methods

Chapter 2

1.0 Cell culture

1.1 Cell lines and reagents

The non-tumourigenic breast epithelial cell line MCF10A was kindly provided by the Bianchi lab, Barts Cancer Institute (BCI). The human breast cancer cell lines BT474, HCC1954, HS578T, MCF7 and MDA-MB-453 were kindly provided by the Schmid lab, BCI. The human breast cancer cell line SKBR3 was kindly provided by the Kermorgant lab, BCI. The human ovarian cancer cell lines Ovcar4, Ov4cis and Ov4carbo were kindly provided by the Lockley lab, BCI. Medium requirements for each cell line are detailed in Table 1. Dulbecco's Modified Eagles medium (DMEM) (REF 41966-029), Roswell Park Memorial Institute medium (RPMI) (REF 21875-034), Fetal Bovine Serum (FBS) (REF 10500-064) and penicillin-streptomycin (REF 15140-122) were all purchased from Gibco. For the MCF10A cell line additional supplements, purchases were as follows: horse serum (Sigma H1138), EGF (Peprotech AF-100-15-1000), hydrocortisone (Sigma H4001-1G), cholera toxin (Sigma C8052) and insulin (Gibco 12585-014).

Table 1. List of cell lines used in project, including cell type and medium requirements.

Cell line	Cell type	Medium requirements
MCF10A	Normal breast	DMEM/F12 +5% horse serum +20ng/mL EGF +0.5ug/mL hydrocortisone +100ng/mL cholera toxin +10ug/mL insulin +1% penicillin-streptomycin
BT474	Breast cancer	RPMI +10% FBS +1% penicillin-streptomycin
HCC1954	Breast cancer	RPMI +10% FBS +1% penicillin-streptomycin
HS578T	Breast cancer	DMEM +10% FBS +1% penicillin-streptomycin
MCF7	Breast cancer	RPMI +10% FBS +1% penicillin-streptomycin
MDA-MB-453	Breast cancer	DMEM +10% FBS +1% penicillin-streptomycin
SKBR3	Breast cancer	DMEM +10% FBS +1% penicillin-streptomycin
Ovcar4	Ovarian cancer, parental	DMEM +10% FBS +1% penicillin-streptomycin
Ov4cis	Ovarian cancer, Cisplatin-resistant	DMEM +10% FBS +1% penicillin-streptomycin
Ov4carbo	Ovarian cancer, Carboplatin-resistant	DMEM +10% FBS +1% penicillin-streptomycin

1.2 Growing and seeding conditions

All cell lines were grown in T75 flasks, at 37°C, 5% CO_2. Cell lines were passaged at 70% confluency and medium was refreshed every 3-4 days. Cell lines were kept up to and including passage 12, from thawing. Stocks of each cell line were made up in FBS + 10% dimethyl sulfoxide (DMSO) (Fisher Chemical D/4121/PB08) and subsequently stored at -80°C or liquid nitrogen. Cells were visually monitored and regularly tested for mycoplasma.

To passage cell lines, old medium was aspirated from the flask and cells were washed with 1x phosphate buffered saline (PBS). Next, 3mL 1x trypsin (Gibco R-001-100) was added to the flask and cells were incubated at 37°C for 3-5min, or until cells detached. 7mL of medium was then added to neutralise the trypsin and cell suspension was centrifuged (1200rpm, 3min). Medium was aspirated from cell pellet and cell pellet was resuspended in 10mL of fresh medium. The required volume of cell suspension was aliquoted into a new flask to grow until the next passage.

For experimental setup, cells were passaged as described. Before cell suspension was returned to a new flask, cells were counted and seeded depending upon required seeding density. A cell counter slide (Invitrogen Countess) was used to count cells, where 10µL of cell suspension was added to the slide to calculate the number of cells in the cell counter (Invitrogen Countess II). An average was calculated based on counts, and cells were seeded for the experiment.

2.0 Protein expression analysis

2.1 Protein extraction

All steps involving protein isolation were carried out on ice. Cells were washed with 1x PBS and lysed using RIPA buffer (150mM NaCl, 1%(v/v) IGEPAL-630 (Sigma I8896-100mL), 0.5%(w/v) sodium deoxycholate (Sigma D6750-25G), 0.1%(w/v) SDS (10% solution Severn Biotech 20-4000-10), 50mM Tris, Protease inhibitor (Roche 11836170001) (1 tablet in 50mL); dissolved in H2O; pH 8). Cells were immediately scraped in the RIPA buffer and the entire well contents was transferred to a microcentrifuge tube, and incubated on ice for a further 5-10min. Samples were then vortexed and centrifuged (12000rpm, 5min, 4°C). The supernatant was transferred to a new microcentrifuge tube and isolated protein was stored at -80°C.

For protein quantification, Bradford reagent (Sigma B6916-500mL) was used to generate a standard curve of bovine serum albumin (BSA) (Sigma A9647-100G) and to subsequently determine the concentration of our protein sample. Absorbance 630 was measured on the plate reader (Fluostar Omega BMG Labtech).

2.2 Western blotting

To prepare samples to load on the gel, 30µg of protein lysate was combined with 10% dithiothreitol (DTT) (Thermo Scientific R0862) and 4x NuPage LDS sample buffer (Invitrogen NP0007) to a final volume of less than 40µL per well. This mix was incubated (5min, 95°C) prior to gel loading. Samples were then pulse centrifuged and electrophoresed following the NuPAGE system (4-12% Bis-Tris protein gels, Invitrogen NP0335BOX) (20x Running buffer, Invitrogen NP0001-02)

for 80min at 130V. A protein ladder (Amersham RPN800E) was run alongside our samples. Gels were transferred onto nitrocellulose membrane (Thermo Scientific 88018), using a wet-transfer method for 2hr at 25V.

Membranes were then blocked with either 5% BSA-TBS-T or 5% milk-TBS-T (1 hour, room temperature) (milk powder, Sigma 70166-500G; 10x TBS, Severn Biotech 20-7301-10; Tween, Fluka P1379-500mL). Next, the membranes were probed with the desired primary antibody (overnight, 4°C), following the dilutions listed in Table 2. The membranes were then incubated in their respective secondary antibody (1:4000, anti-rabbit or anti-mouse), diluted in 5% milk-TBS-T or 5% BSA-TBS-T (1 hour, room temperature). Protein levels were detected by chemiluminescence (SuperSignal West Pico PLUS Chemiluminescent Substrate, Thermo Scientific 34578) on x-ray films. Immunoblotting for β-actin or β-tubulin served as loading controls.

Quantification of Western blots was carried out using ImageJ software for three independent experiments.

Table 2. List of antibodies used in project, including standard dilution.

Antibody name	Company and code	Standard dilution
FOXM1	Abcam ab58675	1:500
FOXM1 (D12D5) XP	Cell Signaling #5436	1:1000
Gpx1	Cell Signaling #C8C4	1:1000
Phospho-NRF2, phospho S40	Abcam ab76026	1:1000
Total NRF2	Abcam ab137550	1:1000
NQO1	Cell Signaling #3187S	1:3000
RAD51	Abcam ab63801	1:1000
SOD1	Cell Signaling #71G8	1:1000
β-actin	Cell Signaling #4970L	1:10000
β-tubulin	Cell Signaling #2146S	1:10000
β-tubulin	Cell Signaling #86298S	1:10000
Secondary rabbit	Dako P0448	1:4000
Secondary mouse	Dako P0447	1:4000

3.0 mRNA expression analysis

3.1 RNA extraction

Cells were lysed with RLT buffer from the RNeasy Mini Kit (Qiagen 74106), and subsequent steps were followed according to the manufacturer's instructions for the kit. To summarise, cells were scraped in the RLT buffer and transferred to a microcentrifuge tube, for each well, and debris was centrifuged (12000rpm, 3min). The supernatant was transferred to a new tube and 70% ethanol was added to lysate. After mixing, the contents were then transferred to an RNeasy Mini spin column with collection tube. The column was centrifuged (8000rpm, 15sec) and the flow-through was discarded. Next, RW1 was added to the column and centrifuged (8000rpm, 15sec). There were then two RPE wash steps at (8000rpm, 15sec) and (8000rpm, 2min), respectively. The columns were then placed into new collection tubes and 50µL RNase-free water was added to each column and left to incubate (10min, room temperature). Once incubated, columns were spun (8000rpm, 1min) and RNA was eluted and collected.

For RNA quantification, the Nanodrop 2000 Spectrophotometer (Thermo Scientific) was used and a blank sample was always measured first before our RNA sample.

3.2 RT-qPCR

The High capacity cDNA reverse transcription kit (Applied Biosystems) was used to prepare cDNA from our total RNA samples. A total of 20µL was made up for each reaction mix, with fixed volumes of 10x buffer (2µL), dNTPS (0.8µL), RT random primers (2µL) and reverse transcriptase (1µL). Upon RNA quantification, 500ng of

RNA was added to each reaction mix, and the final volume of 20µL was made up with RNase-free water. The following Thermocycler reverse transcription settings were used to generate cDNA for each reaction mix: Stage 1 25°C 10min; Stage 2 37°C 60min, 37°C 60min (machine maximum time is 60min so x2 60min set); Stage 3 85°C 5min; and, End 4°C (Applied Biosystems, Veriti 96 well Thermocycler).

For real-time quantitative PCR (RT-qPCR), cDNA (0.5µL) and desired Taqman probe (0.5µL) was added to Taqman Universal PCR Master Mix (Applied Biosystems) (5µL) and H_2O (4µL), for number of wells required. Each sample was assayed in duplicate, where relative mRNA levels were determined according to the ΔΔCt method and subsequently normalised to the level of beta-actin mRNA. RT-qPCR plates were run in QuantStudio 5 (Applied Biosystems). The Taqman probes used are listed in Table 3.

Table 3. List of Taqman probes used in project.

Probe name	Code
BRCA2	Hs00609073_m1
BRIP1	Hs00908143_m1
FOXM1	Hs01073586_m1
MTCO1	Hs02596864_g1
NBS1	Hs01039845_m1
NRF1	Hs01031046_m1
NRF2	Hs00975961_g1
PPARGC1	Hs01016719_m1
RAD51	Hs00947967_m1
β-actin	Hs01060665_g1

4.0 Gene silencing

4.1 6-well plate siRNA transfection

4.1.1 Single siRNA transfection

To obtain protein and RNA from siRNA-transfected cells, cells were seeded in 6-well plates at optimal density on day 1 and transfected with 50nM siRNA (20μM stocks), according to the manufacturer's guide, on day 2. In summary, 250μL optiMEM (Gibco 31985-062) was incubated with 5μL transfection reagent for 5min at room temperature. Then 5μL of required siRNA was added and incubated for

20min at room temperature, per well. Lipofectamine RNAiMAX (Invitrogen 13778-150) was used for siRNA transfection of all cell lines. Medium was aspirated and replaced with fresh medium, in each well. The transfection mix of optiMEM + RNAiMAX + siRNA was added dropwise to each respective well. Medium was refreshed 5-hours post-transfection, in each well. The control siRNA solutions AllStars negative control siRNA (siCon) and positive control Hs_PLK1 siRNA (siPLK1) were used in each experiment. The FOXM1-targeting siRNAs used were: siFOXM1(5) (Qiagen), siFOXM1(6) (Qiagen), and siFOXM1(pool) (Dharmacon). The NQO1-targeting siRNA used was siNQO1(pool) (Dharmacon). Silencing was observed across different time-points (mainly at 48-hours, 72-hours and 96-hours post-transfection for 6- and/or 96-well plate setup).

4.1.2 Double siRNA transfection

The protocol used was similar to single siRNA transfection (section 4.1.1). The difference in this protocol was the amount of siRNA used for each condition in each well. In double siRNA-transfected wells, 5µL of siRNA (50nM final concentration) were used of each siRNA. For wells that contained controls and single siRNA transfections, an additional 5µL of AllStars negative control siRNA was added to account for the two siRNAs used in the double siRNA-transfected well. Besides the amount of siRNA used, the protocol remained the same as Section 4.1.1.

4.2 96-well plate siRNA transfection

4.2.1 Single siRNA transfection

To assess the effect of specific siRNA transfection on cell viability, cells were seeded in 96-well plates at optimal density on day 1 and transfected with 50nM

siRNA (2µM stocks), according to the manufacturer's guidelines, on day 2. In summary, 24.75µL optiMEM was incubated with 0.25µL transfection reagent for 5min at room temperature. Then 2.5µL of required siRNA was added and incubated for 20min at room temperature, per well. The transfection reagent used is as listed in 4.1. Medium was aspirated and replaced with fresh medium, in each well. The transfection mix of optiMEM + RNAiMAX + siRNA was added to each respective well. Medium was refreshed 5-hours post-transfection, in each well. The control siRNA solutions and FOXM1-/NQO1-targeting siRNAs used are as listed in Section 4.1. Silencing was observed across different time-points (mainly at 48-hours, 72-hours and 96-hours post-transfection for 6- and/or 96-well plate setup).

4.2.2 Double siRNA transfection

The protocol used was similar to single siRNA transfection (Section 4.2.1). The difference in this protocol was the amount of siRNA used for each condition in each well. In double siRNA-transfected wells, 2.5µL of siRNA (50nM final concentration) were used of each siRNA. For wells that contained controls and single siRNA transfections, an additional 2.5µL of AllStars negative control siRNA was added to account for the two siRNAs used in the double siRNA-transfected well. Besides the amount of siRNA used, the protocol remained the same as Section 4.2.1.

5.0 Compounds used

All compounds were diluted and stored according to the manufacturer's instructions (Table 4).

Table 4. List of compounds used in project.

Compound name	Reconstituted in, stock	Company and code
Thiostrepton	DMSO, 50mM	Chemcruz sc-203412A
Olaparib	DMSO, 10mM	Cambridge Bioscience CAY10621
Menadione	DMSO, 10mM	Sigma M9429
Podophyllotoxin	Ethanol, 10mM	Sigma P4405
Diminutol	DMSO, 10mM	Santa Cruz Biotechnology sc-203923
Cisplatin	H_2O, 1mg/mL	Adooq Bioscience 15663-27-1
Carboplatin	H_2O, 10mg/mL	Cambridge Bioscience CAY13112

6.0 Cell viability analysis

6.1 Cell-Titer Glo assay

The Cell-Titer Glo reagent (Promega G7573) was diluted 1 in 4 with 1x PBS. Medium was aspirated from each well and 100µL Cell-Titer Glo + PBS mix was added to each well. Next, the plate was put on the plate shaker for 2min at a low speed and then incubated for 10min at room temperature. Luminescence was measured on the plate reader as a measure of cell viability in each well.

7.0 Colony formation analysis

7.1 Sulforhodamine B assay

To assess the colony formation ability of cells upon drug treatment, cells were seeded in 6-well plates at optimal density on day 1, and treated with single agents, combination agents or vehicle control on day 2. The cells were subsequently retreated every 2-3 days, where old medium was removed and fresh drug was added to each well. Colony formation was measured by the sulforhodamine B (SRB) (Sigma S1402-5G) assay, once distinct colonies had formed, on day 10-14.

For the SRB assay, old medium was aspirated and 1x PBS was used to gently wash each well. Next, 1mL of methanol (stored at -20°C) (Fisher M/3950/17) was added to each well to fix the cells, and incubated for 10min at room temperature, and then aspirated off. The wells were then left at room temperature to air dry. Plates could be stored at this step, or proceed directly to SRB staining. For SRB staining, 1mL of SRB stain was added to each well and incubated for 30min at room temperature, and then aspirated off. To wash away excess SRB, 1% acetic acid (Fluka 607-002-00-6) was used to wash each well four times and the plates were finally left to air dry overnight at room temperature. Colonies were either counted by eye or they were dissolved. In the case of counting by eye, a pen was used to mark the bottom of the plate to count each colony. Alternatively, colonies were dissolved in 2mL Tris pH 10.5 and absorbance was read at 490nm on a plate reader.

8.0 Confocal microscopy

8.1 γH2AX staining

To assess the levels of DNA double-strand breaks in our cells upon drug treatment, cells were seeded onto poly-L-lysine-coated coverslips (Corning 354085) in 24-well plates at optimal density on day 1, and treated with agents alone, agents in combination or vehicle control on day 2. Differences in γH2AX foci, a marker of DNA double-strand breaks, which formed upon treatment with agents alone and agents in combination were measured in cancer cells.

At the selected time-point post-treatment, cells were fixed in 4% paraformaldehyde (PFA) (Sigma Aldrich P6148-1KG) 2% sucrose in 1x PBS and incubated for 15min at room temperature. Cells were then washed twice in 1x PBS. Next, 500µL of blocking buffer (1% BSA, 0.1% Tween in 1x PBS) was added to each coverslip. Plates were wrapped in cling film and stored at 4°C until two days before confocal microscopy.

The blocking buffer was then aspirated off the cells, and coverslips were washed twice in 1xPBS. Then 400µL 0.5% Triton (BDH 306324N) in 1x PBS was added to each coverslip and incubated for 10min at room temperature, followed by two 1x PBS washes. The coverslips had blocking with 400µL blocking buffer for 1 hour at room temperature. After blocking, 400µL of primary antibody (γH2AX, 1:800) (Millipore 05-636) in 2% BSA PBS was added. Plates were wrapped in cling film and incubated at 4°C overnight.

The primary antibody was aspirated off the cells, and coverslips were washed three times in 1x PBS at low speed on the plate shaker. Following the washes, 400µL of secondary antibody (anti-mouse ALEXA 488 (green), 1:1000) (Invitrogen A11001) in 2% BSA PBS was added and coverslips were incubated for 1hr, protected from light, at room temperature. Coverslips were then washed three times in 1x PBS. Following the washes, cells were stained with DAPI (1:10000, 1mg/mL) in 1x PBS and incubated for 1min at room temperature. Coverslips were washed twice in 1x PBS and the slides were mounted with Mowiol (5µL/slide) (Calbiochem 475904).

The coverslips were imaged using the confocal LSM 710 microscope (Zeiss), where the 63x oil objective was used to capture images of cells for each condition tested. ImageJ was used to quantify the images and percentages were calculated comparing to the total number of cells (quantified by γH2AX and DAPI staining). Approximately 150 cells were analysed per condition and cells containing more than 5 γH2AX foci were considered positive for DNA double-strand breaks. Previous studies have shown that more than 5 foci per cell are considered positive for DSBs [278, 300, 304, 305], so we selected this to be the cut off in our experiments.

9.0 Reactive oxygen species assay

9.1 DCFDA cellular ROS detection

DCFDA Cellular ROS Detection Assay Kit (Abcam, ab113851) uses 2',7' –dichlorofluorescin diacetate (DCFDA), a fluorogenic dye that enters the cell and measures hydroxyl, peroxyl and other reactive oxygen species activity. DCFDA is first deacetylated by cellular esterases, and this is a non-fluorescent compound. ROS then oxidise this into 2', 7' –dichlorofluorescein (DCF), a highly fluorescent

compound. Fluorescence spectroscopy can be used to detect this fluorescence, with excitation / emission at 485 nm / 535 nm.

To evaluate the generation of ROS upon drug treatment, cells were seeded in two 96-well plates at optimal density on day 1. To measure ROS, a dark, clear bottom 96-well plate was used. The second 96-well plate was used to measure cell viability, in parallel. Cells were treated the same in both 96-well plates, for ROS and cell viability, with the agents alone, the agents in combination or vehicle control on day 2. At our selected treatment end-point, cells in the ROS plate were stained with the cell permeant reagent 2',7' –dichlorofluorescin diacetate (DCFDA), based on the manufacturer's guidelines (Abcam, ab113851, DCFDA Cellular ROS Detection Assay Kit). The cell viability plate was kept in the incubator until measuring the luminescence end-point. In order to assess the level of ROS in the cells, medium was removed from the cells, and cells were then washed with 1x PBS. Cells were then stained with 25μM DCFDA solution and incubated for 45min at 37°C, in the dark. After this incubation, DCFDA solution was removed and this was followed by a 1x PBS wash. After the 1x PBS was aspirated, 1x buffer was added to the cells and incubated for 4 hours at 37°C. Fluorescence was measured on the plate reader Ex/Em = 485/535nm in the ROS plate, and luminescence was measured on the plate reader in the cell viability plate using the CTG assay (Section 6.1). Tert-Butyl Hydrogen Peroxide (TBHP) solution was used as the positive control for ROS, and 1x buffer alone was used as the negative control.

Once the ROS data and CTG data had been normalised to their respective vehicle-treated controls, the ROS data was subsequently normalised to its equivalent CTG data to ensure that reduction in ROS levels was not due to a reduced cell number.

10.0 Drug dose response curves

10.1 Cell viability

Cells were seeded in 96-well plates at optimal density on day 1, and treated with increasing concentrations of the indicated compounds (Table 4) or relevant vehicle control on day 2. Cell viability was measured using Cell-Titer Glo (Section 6.1) on day 5.

11.0 Drug combination analyses

11.1 Cell viability

Cells were seeded in 96-well plates at optimal density on day 1, and treated with agents alone, agents in combination or vehicle control on day 2. For Thiostrepton and Olaparib drug combination analyses, cells were subsequently retreated on day 7, where medium was removed and fresh drug in medium was added to each well. CTG was measured on day 10 (Section 6.1). For Thiostrepton and Diminutol drug combination analyses, cells were not retreated and CTG was measured on day 6 (Section 6.1). For Thiostrepton and Menadione drug combination analyses, cells were retreated on day 7 and CTG was measured on day 10 (Section 6.1).

11.2 Colony formation

Cells were seeded in 6-well plates at optimal density on day 1, and treated with agents alone, agents in combination or vehicle control on day 2. The cells were subsequently retreated on day 5 and day 8, where medium was removed and fresh

drug in medium was added to each well at each retreatment. Colony formation was measured on day 11 (Section 7.1). Colonies were counted by eye or dissolved.

11.3 γH2AX staining

Cells were seeded in 24-well plates at optimal density on day 1, and treated with agents alone, agents in combination or vehicle control on day 2. For Thiostrepton and Olaparib drug combination analyses, coverslips were fixed on day 3, 24-hours post-treatment. For Thiostrepton and Diminutol drug combination analyses, coverslips were fixed on day 4, 48-hours post-treatment (Section 8.1).

11.4 DCFDA cellular ROS detection

Cells were seeded in a cell viability 96-well plate and a ROS dark, clear bottom 96-well plate, at optimal density on day 1, and treated with agents alone, agents in combination or vehicle control on day 2. For Thiostrepton and Diminutol drug combination analyses, cells were DCFDA-stained on day 2, 3-hours post-treatment (Section 9.1).

12.0 Bioinformatics

Using the TCGA-BRCA database, breast cancer RNA-seq data was used to generate “survival: median dichotomisation” graphs, where tumour samples was represented as n=1,092. These analyses were performed independently for low expression and high expression of FOXM1, and also for RAD51.

13.0 Data analysis and representation

All graphs were generated using Prism software. In graphs where multiple repeats were represented, error bars were plotted as standard error of the mean (SEM). In graphs where a single repeat was represented, standard deviation (SD) was plotted for error bars.

As a t-test is used to determine if there is a significant difference between the means of two groups, which may be related in certain features, we considered this test to be the most appropriate to be used in our data analyses. Therefore, a t-test was used for analysis between two sets of data, where two-sided, unpaired tests were carried out. When a t-test showed statistical significance, the p-value was listed as <0.05*, <0.01**, <0.001***, or <0.0001****, depending upon the level of significance.

Results

Chapter 3 – Investigating Thiostrepton and Olaparib as a combination therapy and its mechanistic implications

Chapter 4 – Investigating the role of FOXM1 in the oxidative stress response and its therapeutic implications

Chapter 3 - Investigating Thiostrepton and Olaparib as a combination therapy and its mechanistic implications

1.0 Basal FOXM1 and RAD51 expression levels in cancer

1.1 Expression levels of FOXM1 and the homologous recombination protein RAD51 vary in different cancer cell lines

Previous studies have indicated *Foxm1* as a candidate gene for targeted therapy and diagnostics in cancer, and it was found to be highly overexpressed in almost every tumour analysed [180]. FOXM1 overexpression has been strongly correlated with poor prognosis in breast cancer [168]. As FOXM1 is known to play a role in regulating DNA repair genes [115], this may offer a target for a combined therapy in cancer cells. The potential of exploiting synthetic lethality in cancer cells with FOXM1 overexpression has already been shown in a similar premise, where drug-resistant glioblastoma cells were sensitised to Temozolomide through FOXM1 inhibition, and a subsequent downregulation of the homologous recombination protein, RAD51 [116]. Furthermore, *Rad51* was identified to be under the transcriptional regulation of FOXM1, where re-expression of RAD51 in FOXM1-depleted cells gave partial rescuing of Temozolomide resistance [116]. In this study, we aimed to develop a combination therapy, to target FOXM1 and a reduced

homologous recombination pathway, as well as to understand the mechanistic basis for this potential combination treatment.

We first determined whether basal FOXM1 levels varied in our cell line panels which included breast cancer, ovarian cancer, mesothelioma, bladder cancer and colorectal cancer. In recurrent glioblastoma multiforme tumour samples, FOXM1 expression has previously been directly correlated with RAD51 expression [116]. Therefore, we next assessed basal expression levels of RAD51 in each of our cell line panels, so that we could identify if FOXM1 levels correlated with RAD51 levels in other cancer types. The non-tumourigenic breast epithelial cell line MCF10A was also included in the breast cell line panel to compare against the breast cancer cell lines. Expression levels of MCF10A cells is therefore important to consider when developing therapeutic options for cancer patients, in order to spare their normal cells.

For the breast cancer cell line panel, upon quantification of the Western blots (Figure 8A(i)), MDA-MB-453 and BT474 showed that when FOXM1 expression level was high, RAD51 expression level was also high. The opposite was observed for MCF7 and HS578T, such that when FOXM1 expression level was low, RAD51 expression level was also low. MCF10A and HCC1954 had variation in the different isoforms of FOXM1, but both cell lines had high RAD51 expression levels.

For the ovarian cancer cell line panel, upon quantification of the Western blots (Figure 8B(i)), one FOXM1 isoform (bottom band) was more greatly expressed than the other (top band) across all cell lines. The lowest expression of FOXM1 (top band) also correlated with the lowest expression of RAD51 in PEO4.

For the mesothelioma and bladder cancer cell line panel, upon quantification of the Western blots (Figure 8C(i)), the FOXM1 isoform (bottom band) was expressed lower in Ju77S, compared to other mesothelioma cell lines. Whilst the other FOXM1 isoform (top band) was expressed higher in MSTO compared to other mesothelioma cell lines. FOXM1 expression levels do not seem to correlate with RAD51 expression levels in mesothelioma or bladder cancer cell lines.

For the colorectal cancer cell line panel, upon quantification of the Western blot (Figure 8D(i)), both of the FOXM1 isoforms were expressed higher in SW48 and SW620, compared to the other cell lines. FOXM1 expression levels do not seem to correlate with RAD51 expression levels in colorectal cancer cell lines.

As this experiment is not N=3 this is a caveat, and must be taken into consideration when making conclusions. This also applies to later experiments that are not N=3.

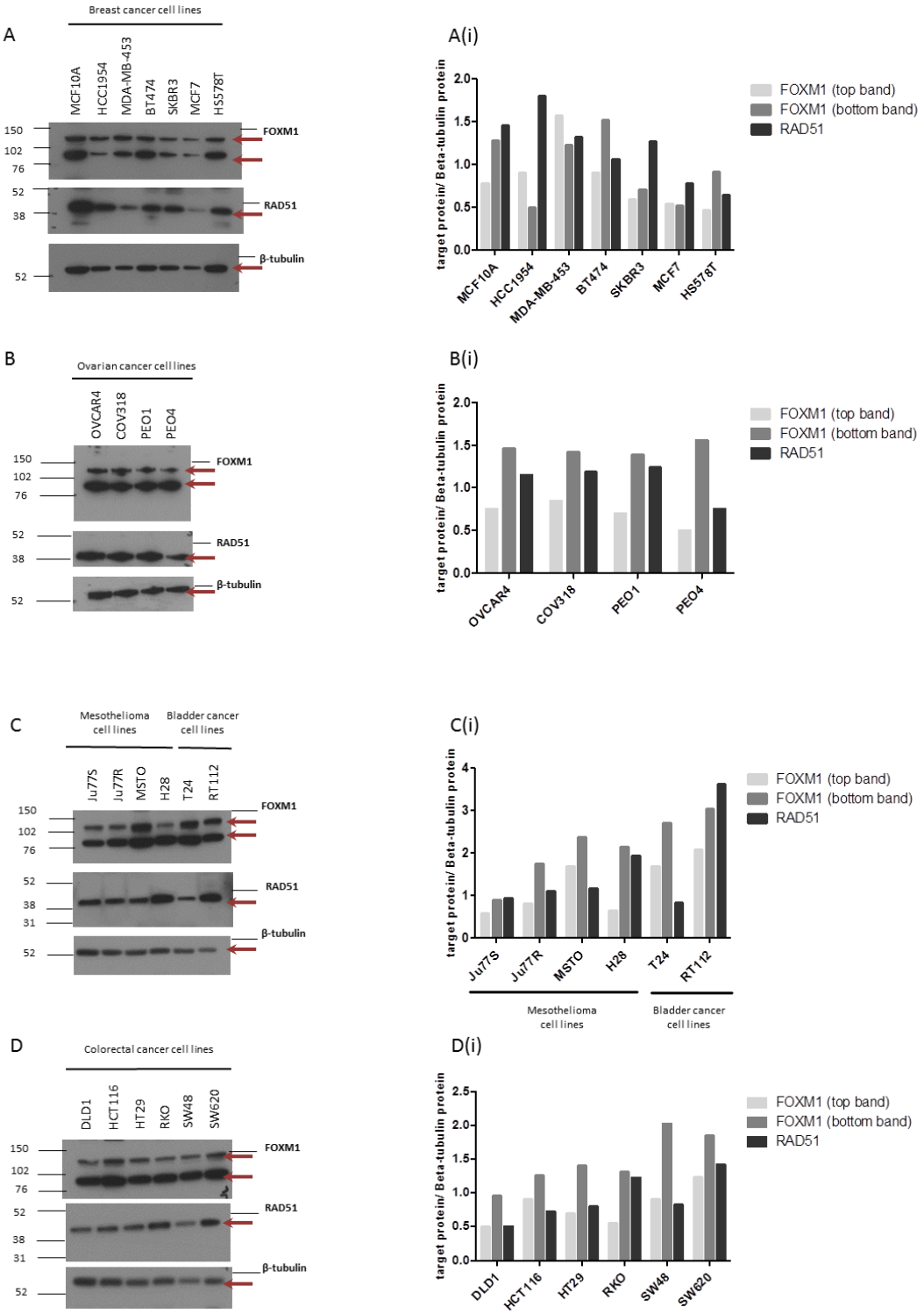


Figure 8. FOXM1 and RAD51 expression in cancer cell line panels.

Whole cell lysates were isolated for a panel of (A) (Ai) breast cancer cell lines, (B) (Bi) ovarian cancer cell lines, (C) (Ci) mesothelioma and bladder cancer cell lines, and (D) (Di) colorectal cancer cell lines, and analysed on a Western blot and probed with FOXM1 and RAD51 antibodies. β-tubulin was used as a loading control. N=1. Image J was used to quantify Western blots.

We used our breast cancer cell lines as a model for the rest of our study on targeting FOXM1 and homologous recombination. This is because FOXM1 and RAD51 expression showed correlation in some breast cancer cell lines (Figure 8A), and these differences may reflect the effectiveness of a therapy for treating cancer patients.

We selected a range of breast cancer cell lines in this project, to have a representative cell line from a number of different breast molecular subtypes. The breast cancer cell lines HCC1954, BT474, and SKBR3, are all in the HER+ subtype. The HS578T breast cancer cell line is Triple Negative Breast Cancer (TNBC) subtype, Basal B subgroup. While, the MDA-MB-453 breast cancer cell line is TNBC subtype, Basal A subgroup. The MCF7 breast cancer cell line is the only ER+ subtype, Luminal A subgroup. Finally, the MCF10A human breast epithelial cell line was selected as the model of “normal”, as one of the most commonly used normal breast cell models [306]. The MCF10A cell line was ultimately, upon our investigation, not an optimal model of normal in terms of FOXM1 expression, because FOXM1 is highly expressed in this cell line. Then, normal cells in a cancer patient may need low expression levels of FOXM1 to have a tolerable toxicity to FOXM1 inhibition [183]. Furthermore, when the MCF10A cell line was previously evaluated as a reliable model for normal human mammary epithelial cells, the results did challenge the model [306]. This study showed that there are differences in marker expression profiles between MCF10A cells in 2D culture as well as 3D culture, compared to normal mammary tissue [306]. Therefore, the MCF10A cell line may not be a suitable model for normal breast epithelial cells, in general.

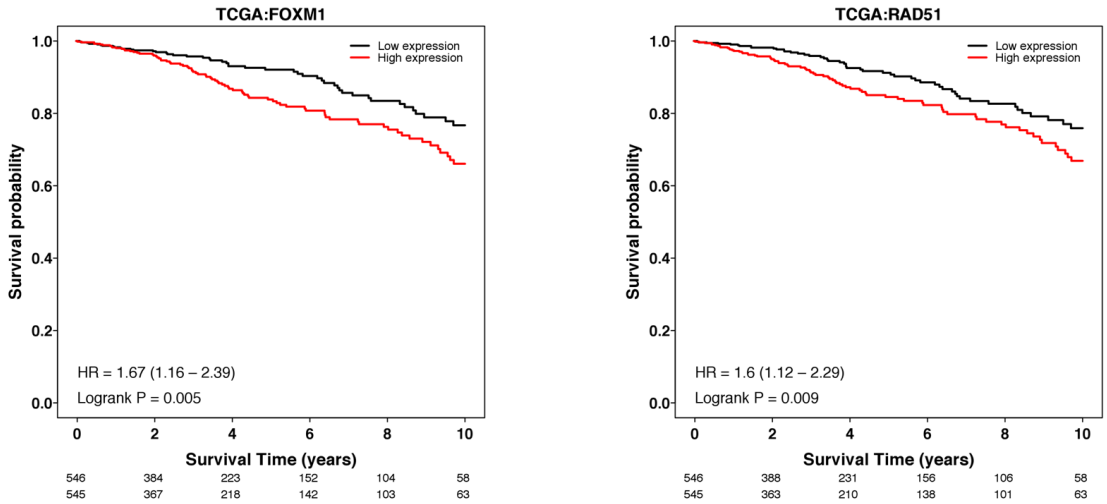


Figure 9. High expression of FOXM1 and high expression of RAD51 correlate with reduced survival probability.

Using The Cancer Genome Atlas (TCGA) database, patients were compared for low and high expression of FOXM1 and RAD51, independently (P>0.05 for multivariate analysis).

We also investigated whether this relationship between FOXM1 and RAD51 translated beyond our initial *in vitro* studies, and we carried out bioinformatics analysis using breast cancer patient data from the TCGA database. From this broad analysis, we established that there was a significant correlation between patients that had either high FOXM1 expression or high RAD51 expression, and a reduced survival probability (Figure 9). Further investigation is required to determine whether those patients with high FOXM1 expression levels also have high RAD51 expression levels.

2.0 Analysing the sensitivity of breast cancer cells to Thiostrepton and to Olaparib

2.1 Treatment with the FOXM1 inhibitor Thiostrepton and the PARP inhibitor Olaparib reduced cell viability

Based on our data demonstrating FOXM1 and RAD51 correlate in breast cancer cells (Figure 8A), and that this also seems to be evident in breast cancer patients (Figure 9), we subsequently explored the therapeutic strategy of targeting FOXM1 and a reduced homologous recombination pathway. To this end, we used the FDA-approved FOXM1 inhibitor, Thiostrepton, and the FDA-approved PARP inhibitor, Olaparib, in our breast cancer cells.

The PARP inhibitor Olaparib is FDA-approved for clinical treatment of patients with mutations in HR genes, *Brca1* and *Brca2*, in ovarian cancer and, more recently, breast cancer [39, 40]. Olaparib is a targeted treatment that causes cell death of cancer cells only, sparing normal cells, through synthetic lethality [307]. Inhibition of PARP, by Olaparib, combined with a mutation in the cancer cell such as *Brca1*-deficiency, will together cause cancer cell death [307]. Based on these current uses of Olaparib, this is what made it the ideal compound to be used in combination with Thiostrepton in our study. Thus, on the basis of the role of FOXM1 in the DNA damage response and the mechanism of action of Olaparib, we hypothesised that inhibiting FOXM1 will then reduce the levels of essential DNA repair HR proteins, including RAD51, and sensitise these cells to Olaparib.

We first determined whether treatment with Thiostrepton and Olaparib alone caused any effect on cell viability in our breast cancer cells. We treated cells with either increasing concentrations of the FOXM1 inhibitor Thiostrepton (Figure 10A-D) or the PARP inhibitor Olaparib (Figure 11A-F) for 72-hours, and analysed cell viability.

Initially, a range of Thiostrepton and Olaparib concentrations were tested and this was modified according to cell sensitivity. For subsequent experiments, these ranges also helped selection of concentrations that would not be too toxic to the cell for the type of experiment.

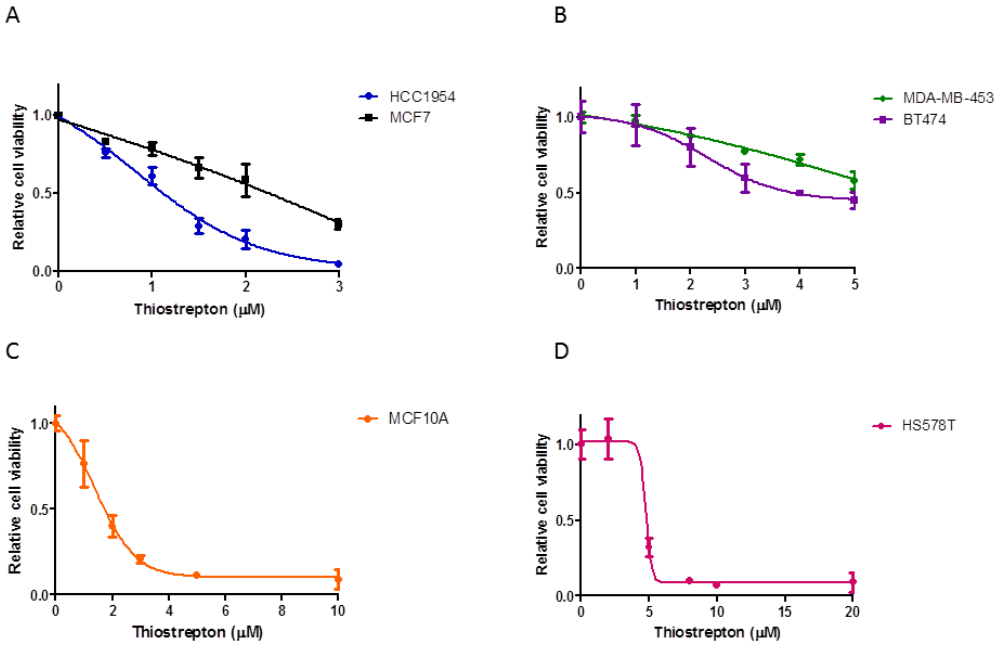


Figure 10. Sensitivity of a panel of breast cancer cell lines, and MCF10A, to Thiostrepton treatment.

A panel of breast cancer cells A) HCC1954 and MCF7, B) MDA-MB-453 and BT474, C) MCF10A, and D) HS578T, were plated in a 96-well plate and treated with increasing concentrations of Thiostrepton, as indicated. Cell viability was analysed using an ATP-luminescent assay 72-hours post-treatment. Data was normalised to vehicle (DMSO) control. N=2 (HCC1954 and MCF7) and error bars represent SEM. N=1 (MDA-MB-453, BT474, MCF10A, HS578T) and error bars represent SD.

Table 5. IC50 values for breast cancer cell lines, and MCF10A, treated with Thiostrepton.

Cell line	IC50 value (µM)
HCC1954	1.3
MCF7	1.9
MDA-MB-453	Out of range
BT474	2.5
HS578T	4.3
MCF10A	1.8

Our results suggested that our breast cancer cell lines and our non-tumourigenic breast epithelial cell line MCF10A responded in a similar therapeutic window to the FOXM1 inhibitor, Thiostrepton, up to around 5µM (Figure 10A-D). The breast cancer cell lines HS578T (IC50 4.3µM, Table 5), MDA-MB-453 (IC50 out of range, Table 5) and BT474 (IC50 2.5µM, Table 5) were the least sensitive to Thiostrepton, compared to the other breast cancer cell lines. Interestingly, MDA-MB-453 and BT474 cells showed some of the highest expression levels of FOXM1 (Figure 8A), so it could be that these cells are still able to resist Thiostrepton at this particular concentration. In contrast, our non-tumourigenic breast epithelial MCF10A cells and our HCC1954 breast cancer cells were the most sensitive cell lines to Thiostrepton (Table 5). These cell lines also showed some of the higher expression levels of FOXM1 (Figure 8A), so it could be that these cells are more dependent on FOXM1 for cell viability.

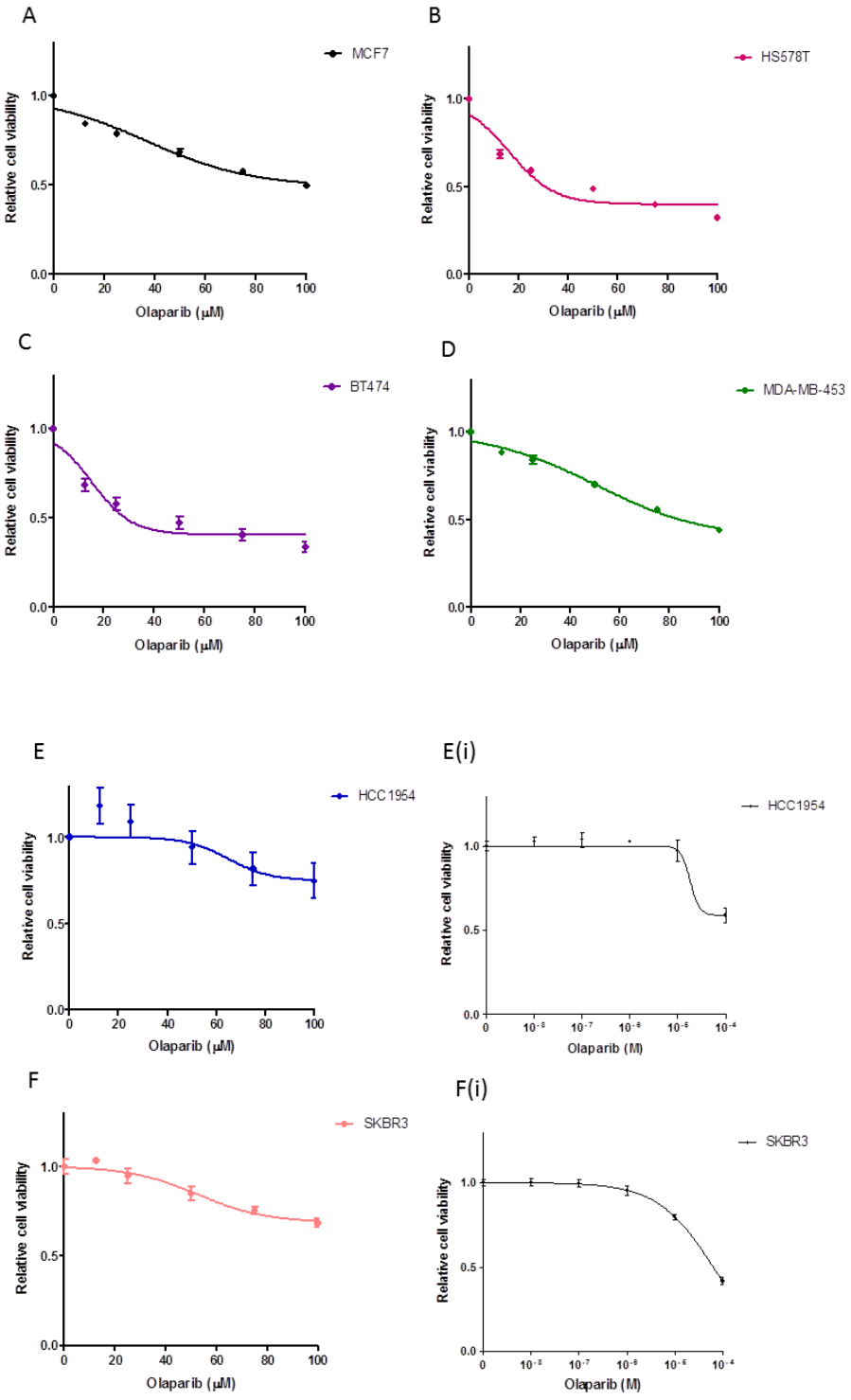


Figure 11. Sensitivity of a panel of breast cancer cell lines to Olaparib treatment.

A panel of breast cancer cells A) MCF7, B) HS578T, C) BT474, D) MDA-MB-453, E) Ei) HCC1954, and F) Fi) SKBR3, were plated in a 96-well plate and treated with increasing concentrations of Olaparib, as indicated. Cell viability was analysed using an ATP-luminescent assay 72-hours post-treatment. Data was normalised to vehicle (DMSO) control. A-D N=3, E-F N=2 (including a third repeat, Ei and Fi, across a different concentration range).

Table 6. IC50 values for breast cancer cell lines treated with Olaparib.

Cell line	IC50 value (μM)
HCC1954	Out of range
MCF7	Out of range
SKBR3	Out of range
MDA-MB-453	83.3
BT474	25.1
HS578T	28.3

As with Thiostrepton, our results suggested that our breast cancer cell lines seemed to respond across a similar therapeutic window to the PARP inhibitor, Olaparib (Figure 11A-F). The breast cancer cell lines MCF7, SKBR3 and HCC1954 (IC50 values all out of range, Table 6) were the least sensitive Olaparib, compared to the other breast cancer cell lines. The SKBR3 and HCC1954 cell lines had higher expression levels of RAD51, compared to MCF7 (Figure 8A). Therefore, with less basal RAD51 expression MCF7 cells may respond to Olaparib through a different mechanism, compared to SKBR3 and HCC1954 cells. Whereas, SKBR3 and HCC1954 cells may resist Olaparib because of their existing higher basal expression levels of RAD51 and this may already compensate for the PARP inhibition that is exerted on these cells. Conversely, HS578T and BT474 cells were the most sensitive to Olaparib (Table 6). These cell lines showed some of the lower expression levels of RAD51 (Figure 8A), so this could be the reason that these cells are more sensitive to PARP inhibition. Therefore, lower RAD51 expression levels combined with PARP inhibition may be what causes these cells to exhibit reduced cell viability.

Taken together, our results suggested that FOXM1 status and RAD51 status of our breast cancer cell lines did not necessarily influence the response to the FOXM1 inhibitor Thiostrepton and the PARP inhibitor Olaparib. This may also suggest that a

combined therapy of Thiostrepton and Olaparib could be used to treat a broader group of cancer patients, independent of their expression levels of FOXM1 and/or RAD51.

3.0 A combined treatment of Thiostrepton and Olaparib show they more greatly reduce viability in breast cancer cells

3.1 Thiostrepton and Olaparib treatment in combination cause significantly reduced cell viability, compared to either agent alone

The concept of targeting FOXM1 as part of a combination therapy has been investigated using a number of approaches. A previous study has indicated that targeting FOXM1 provides a strategy for sensitising ovarian cancer cells to Cisplatin, and it was suggested that this is partially mediated by changes in a downstream target of FOXM1, the DNA repair gene EXO1 [308]. During the time that we were conducting our study, other groups published data showing how they had been working on similar strategies to us. One study demonstrated that the addition of Thiostrepton to Paclitaxel and Cisplatin showed synergistic effects in chemoresistant ovarian cancer cells [309]. Another group also showed that silencing *Foxm1*, followed by treatment with a number of chemotherapeutic agents, including Carboplatin and Olaparib, significantly enhanced the response to these chemotherapeutic agents in ovarian cancer cells [190]. An even more recent study has shown that Thiostrepton enhances sensitivity to Olaparib, through a suggested mechanism of decreased *Brca1* and *Brca2* that the authors described as

"BRCAness", in ovarian cancer cells [310]. Collectively, these studies provide evidence that the combination therapy of Thiostrepton and Olaparib could be a promising treatment option to offer ovarian cancer patients. We therefore tested this combination therapy in our breast cancer cells, to determine whether Thiostrepton and Olaparib could also be used in the treatment of breast cancer patients.

To investigate this, we treated HCC1954, MCF7, MDA-MB-453 and HS578T breast cancer cells, on day 2 and day 7, with Thiostrepton alone, Olaparib alone, Thiostrepton and Olaparib in combination, or vehicle control, and analysed cell viability on day 10.

Thiostrepton and Olaparib concentrations used were from the lower end of the range in Figure 10 and Figure 11 to ensure low toxicity for these experiments. These concentrations were also selected based on a study which showed that Thiostrepton inhibits FOXM1 expression, 10µmol/L Thiostrepton was used to reduce FOXM1 expression over a 24-hour period in MCF7 breast cancer cells [183]. In a more recent study, 24-hour treatment with 3.75µM of Thiostrepton was shown to reduce FOXM1 expression levels in MCF7 breast cancer cells [311]. Based on these studies of MCF7 breast cancer cells treated with Thiostrepton, there seems to be a range of concentrations that can be selected to ensure the target, FOXM1, is inhibited in breast cancer cell lines. For Olaparib, IC50 values of 5 nM, 1 nM and 4 nM for PARP1, PARP2 and PARP3 respectively have been reported, and a study has shown that 1µM Olaparib inhibited PARP [312]. Moreover, following on from this, we optimised the cell viability assays based on the toxicity of drugs.

For this figure, and all successive figures with statistical analyses, the standard error of the mean of three biological repeats was calculated, performed in triplicate. Where triplicate was not carried out, this is noted in the figure legend.

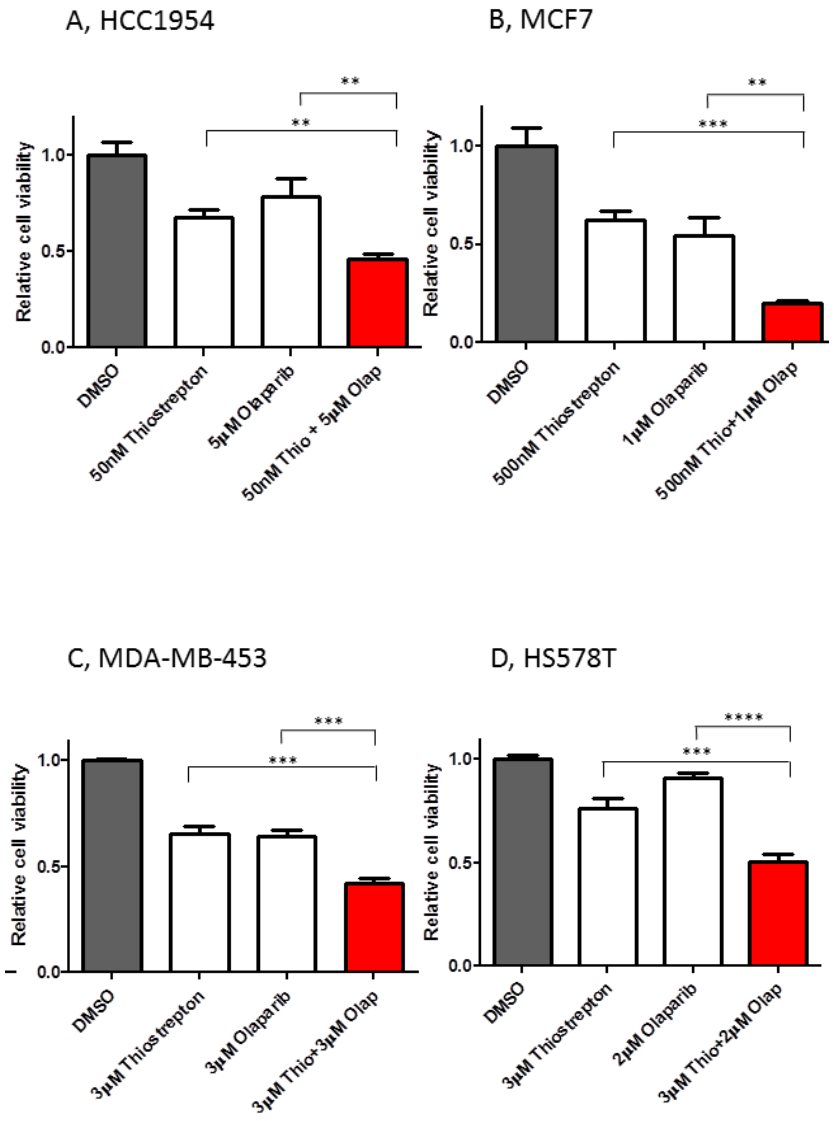


Figure 12. Treatment with Thiostrepton and Olaparib in combination cause a greater reduction in cell viability, compared to either agent alone, in breast cancer cells.

A panel of breast cancer cells A) HCC1954, B) MCF7, C) MDA-MB-453, and D) HS578T, were plated in a 96-well plate and treated with Thiostrepton alone (white), Olaparib alone (white), Thiostrepton and Olaparib in combination (red), or DMSO-vehicle control (grey), as indicated. Cell viability was analysed using an ATP-luminescent assay on day 10 post-treatment. The treatments used were: HCC1954,

50nM Thiostrepton alone, 5µM Olaparib alone, 50nM Thiostrepton + 5µM Olaparib and vehicle control, DMSO; MCF7, 500nM Thiostrepton alone, 1µM Olaparib alone, 500nM Thiostrepton + 1µM Olaparib and vehicle control, DMSO; MDA-MB-453, 3µM Thiostrepton alone, 3µM Olaparib alone, 3µM Thiostrepton + 3µM Olaparib and vehicle control, DMSO; and, HS578T, 3µM Thiostrepton alone, 2µM Olaparib alone, 3µM Thiostrepton + 2µM Olaparib and vehicle control, DMSO. Data was normalised to its vehicle (DMSO) control. N=3 and error bars represent SEM. T-tests were used (p-value of: <0.01**, <0.001***, <0.0001****).

Our results were consistent with what has been shown previously in ovarian cancer cells [310], and suggest that treatment with Thiostrepton and Olaparib in combination caused a greater reduction in cell viability, compared to either agent alone, for the first time in breast cancer cells (Figure 12A-D). Furthermore, all breast cancer cell lines showed that Thiostrepton and Olaparib in combination caused a greater reduction in cell viability, compared to either agent alone, indicating that this response was not FOXM1- or RAD51-expression level dependent.

There was no measurement of drug potency in cell lines in this particular experiment, which limits full interpretation (Figure 12). This applies to later experiments, also lacking this measurement.

3.2 Thiostrepton and Olaparib treatment in combination cause significantly reduced colony formation, compared to either agent alone

To corroborate our findings suggesting that Thiostrepton and Olaparib in combination cause a greater reduction in cell viability, compared to either agent alone, in breast cancer cells, we also analysed treatment with Thiostrepton and Olaparib in combination by clonogenic assay. To this end, we treated HCC1954, MCF7, MDA-MB-453 and HS578T breast cancer cells on day 2, day 5, and day 8

with Thiostrepton alone, Olaparib alone, Thiostrepton and Olaparib in combination, or vehicle control.

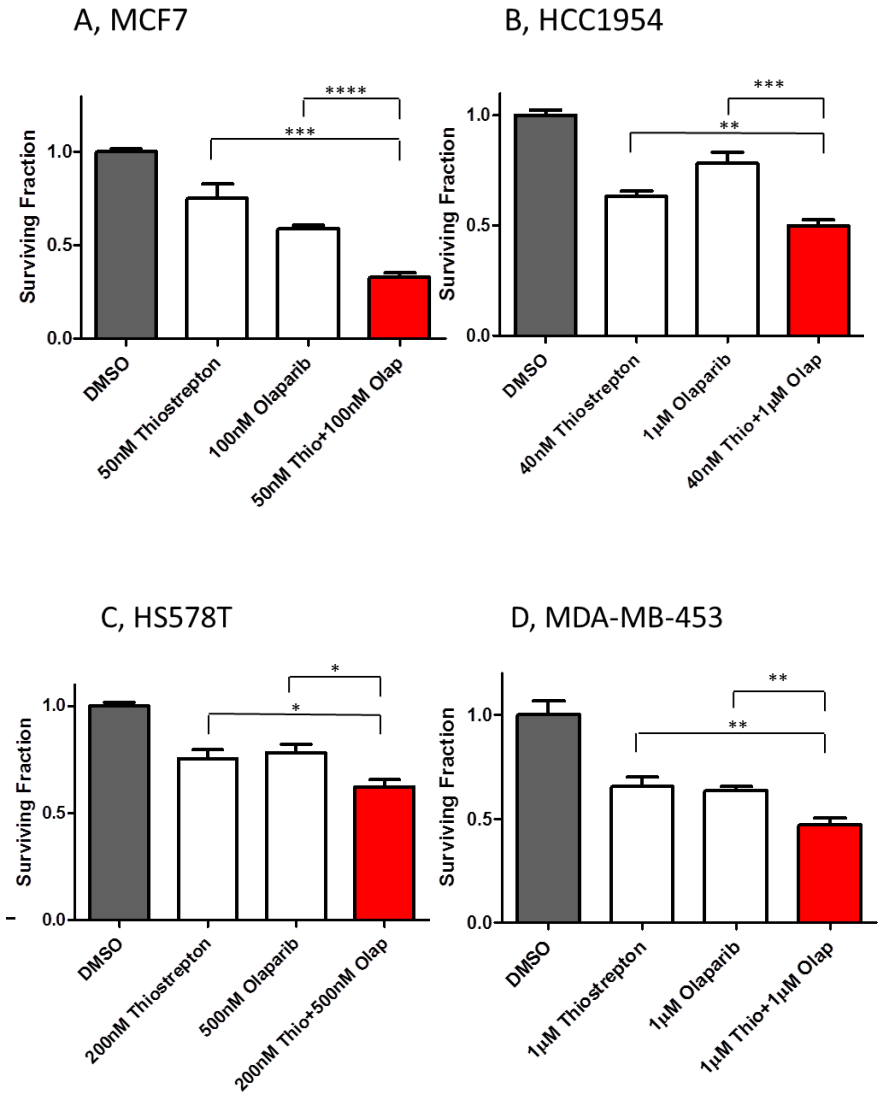


Figure 13. Treatment with Thiostrepton and Olaparib in combination cause a greater reduction in colony formation, compared to either agent alone, in breast cancer cells.

A panel of breast cancer cells A) MCF7, B) HCC1954, C) HS578T, and D) MDA-MB-453, were plated in a 6-well plate and treated with Thiostrepton alone (white), Olaparib alone (white), Thiostrepton and Olaparib in combination (red), or DMSO-vehicle control (grey), as indicated. Colony formation was analysed using SRB staining and counting colonies, on day 11 post-treatment. The treatments used were: MCF7, 50nM Thiostrepton alone, 100nM Olaparib alone, 50nM Thiostrepton + 100nM Olaparib and vehicle control, DMSO; HCC1954, 40nM Thiostrepton alone, 1µM Olaparib alone, 40nM Thiostrepton + 1µM Olaparib and vehicle control, DMSO; HS578T, 200nM Thiostrepton alone, 500nM Olaparib alone, 200nM Thiostrepton + 500nM Olaparib and vehicle control, DMSO; and, MDA-MB-453,

1μM Thiostrepton alone, 1μM Olaparib alone, 1μM Thiostrepton + 1μM Olaparib and vehicle control, DMSO. Data was normalised to its vehicle (DMSO) control sample. N=3 and error bars represent SEM. T-tests were used (p-value of: <0.05*, <0.01**, <0.001***, <0.0001****).

We analysed colony formation on day 11, confirming our cell viability analysis that Thiostrepton and Olaparib in combination caused a more significantly reduced colony formation, compared to either agent alone, in breast cancer cells (Figure 13A-D). Again, all breast cancer cell lines showed a significantly greater reduction in colony formation when treated with Thiostrepton and Olaparib in combination, indicating that this response was not FOXM1- or RAD51-expression level dependent.

Having observed that Thiostrepton and Olaparib in combination more greatly reduced cell viability and colony formation in breast cancer cells, compared to either agent alone, we have therefore provided significant evidence that this combination therapy can be more widely exploited than what has been currently suggested for ovarian cancer patients [310]. We have therefore shown that Thiostrepton and Olaparib could also have important therapeutic implications in the treatment of breast cancer patients, independent of molecular subtype.

4.0 Depletion of FOXM1 alters expression levels of homologous recombination proteins

4.1 Cell viability is reduced upon *Foxm1* silencing in breast cancer cells

To understand the mechanistic basis for Thiostrepton and Olaparib as a combination therapy in breast cancer cells, we next investigated the levels of the range of HR genes using siRNA targeting *Foxm1* and FOXM1 inhibition using Thiostrepton. Prior to investigating the effect of *Foxm1* silencing on members of the homologous recombination pathway, we first determined the effect of *Foxm1* silencing on cell viability in our breast cancer cells. We transfected HCC1954 and MCF7 cells with either siRNA against FOXM1 (si*Foxm1*(6); si*Foxm1*(pool)) and a non-targeting siRNA control (siCon) for 72- and 96-hours, and then measured cell viability.

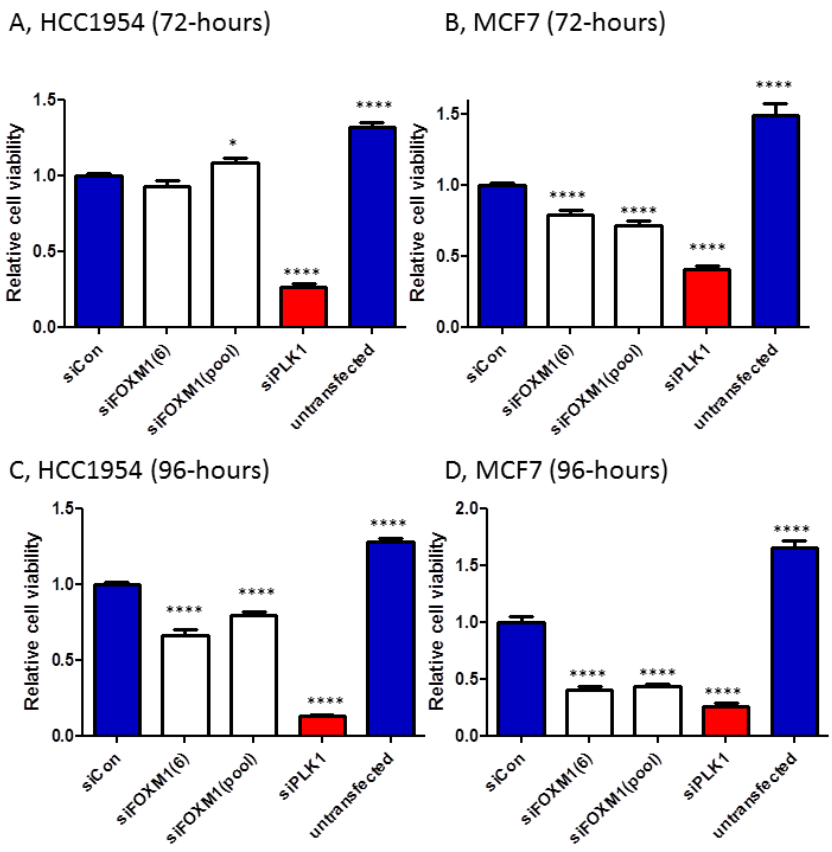


Figure 14. Foxm1 silencing causes reduced cell viability in breast cancer cells.

Cell viability of the breast cancer cell lines HCC1954 and MCF7, plated in a 96-well plate, was analysed A) B) 72- and C) D) 96-hours post-transfection with the FOXM1-targeting siRNAs, si*Foxm1*(6) and si*Foxm1*(pool), using an ATP-luminescent assay. The controls used for this transfection were siCon (negative control) and siPLK1 (positive control). N=3 and error bars represent SEM. T-tests were used (p-value of: <0.05*, <0.01**, <0.001***, <0.0001****).

Our results showed that depletion of FOXM1 resulted in a significant reduction in cell viability in our cell lines, and this was to a greater extent at 96-hours post-transfection (Figure 14A-D). Breast cancer cells may therefore have a threshold of expression of FOXM1 that is required for viability, in particular when FOXM1 is depleted for a greater amount of time. Our *Foxm1* silencing data (Figure 14A-D) therefore correlated with our Thiostrepton sensitivity data (Figure 10A), for the HCC1954 and MCF7 cells, where FOXM1 inhibition also reduced cell viability. It

must also be noted that, there was no measurement of efficiency of siRNA in this experiment, which limits full interpretation.

4.2 FOXM1 regulates the expression levels of a number of genes involved in homologous recombination in breast cancer cells

We next explored the extent at which a number of genes involved in homologous recombination may be regulated by changes in FOXM1 expression, and could therefore be involved in mediating Olaparib sensitisation upon Thiostrepton treatment, in our breast cancer cells. We performed RT-qPCR on the breast cancer cell lines MCF7, HCC1954 and HS578T that were transfected with two different siRNAs targeting FOXM1 (si*Foxm1*(5) and si*Foxm1*(pool)) or a non-targeting siRNA control (siCon; Figure 15A-C). We measured the expression levels of a range of genes shown to be involved in homologous recombination. These included BRCA2; mediates the recruitment of the recombinase RAD51 to DNA double-strand breaks, BRIP1; acts with BARD1 to interact with BRCA1 to organise repair proteins on this BRCA1 scaffold, NBS1; part of the MRN complex, which resects the DNA to form 3′ overhangs that are bound by RPA, and RAD51; the RAD51 nucleoprotein filament is involved in strand invasion, allowing the remaining DNA repair to occur with the use of the sister chromatid as a template for error-free repair.

si*Foxm1*(6) and si*Foxm1*(pool) were initially tested in cell viability assays (Figure 14). si*Foxm1*(5) was later tested in RT-qPCR assays (Figure 15). When compared, si*Foxm1*(5) and si*Foxm1*(pool) showed the most consistent reduction in *Foxm1*

expression across numerous experiments and therefore were used in subsequent experiments in this project.

Our results showed that, upon *Foxm1* silencing (Figure 15A-D), we observed significant changes in the expression of *Brca2*, *Brip1*, *Nbs1*, and *Rad51*. Furthermore, we validated previous findings that show FOXM1 as a transcriptional regulator of *Brca2*, *Brip1*, and *Nbs1*, in breast cancer cells [115, 117, 118]. We also validated previous findings that show FOXM1 as a transcriptional regulator of *Rad51*, in glioblastoma cells [116]. Significantly, we have shown for the first time that FOXM1 regulates *Rad51* expression in breast cancer cells. Furthermore, the expression of *Rad51* and *Brip1* were the most consistently reduced in all three breast cancer cell lines, compared to *Brca2* and *Nbs1* expression, upon *Foxm1* silencing. This data suggests that RAD51 and BRIP1 may be playing a more direct role in mediating Olaparib sensitisation upon Thiostrepton treatment, in breast cancer cells. If this is the case, this mechanism differs to what has been suggested for ovarian cancer cells, where “BRCAness” is described, through decreased expression of *Brca1* and *Brca2* [310].

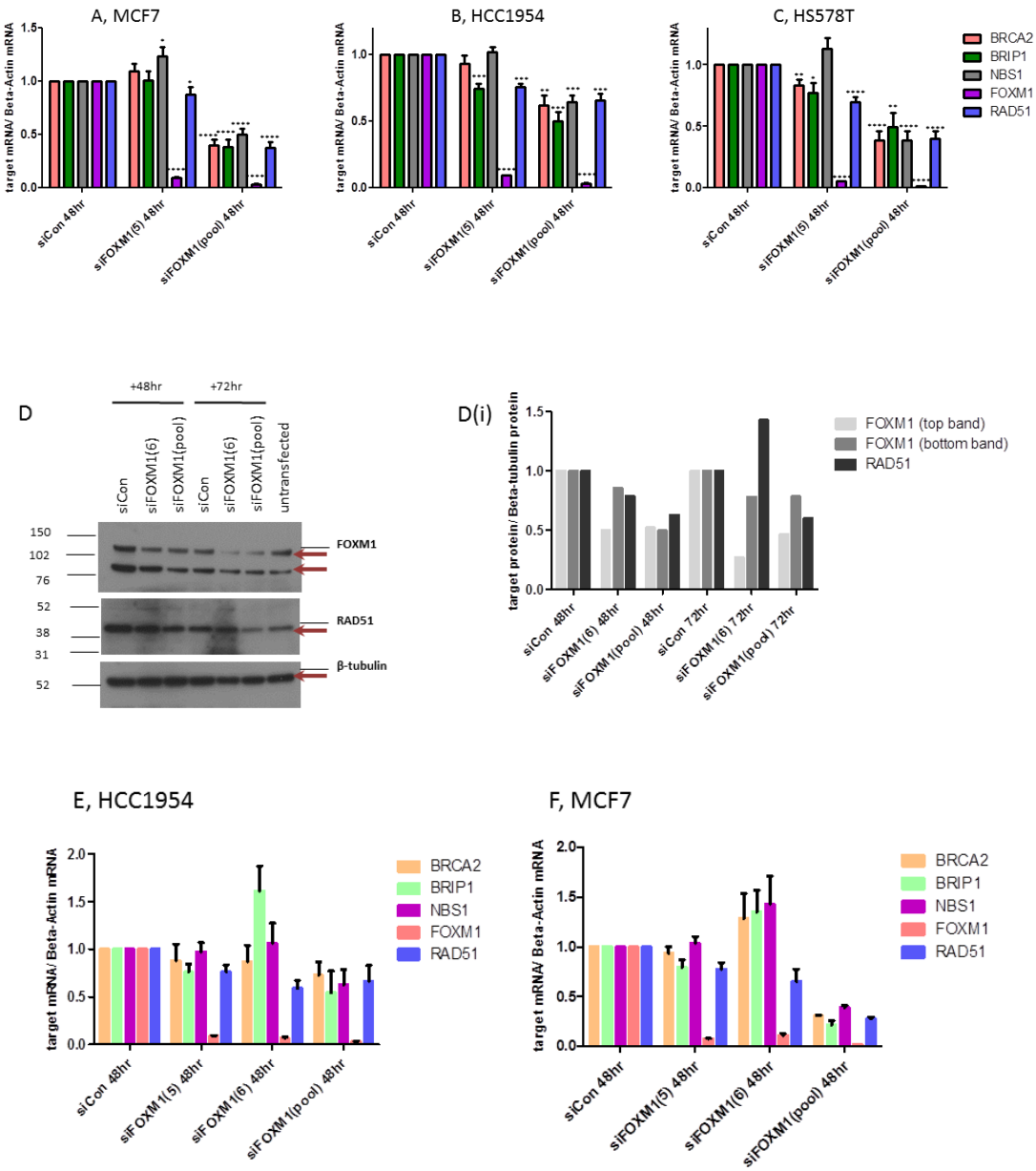


Figure 15. FOXM1 regulates the expression levels of a number of genes involved in homologous recombination in breast cancer cells.

RNA was collected from the breast cancer cell lines A) MCF7, B) HCC1954, and C) HS578T, 48-hours post-transfection with si*Foxm1*(5) and si*Foxm1*(pool) and RT-qPCR performed. β-actin was used as a control and mRNA levels of BRCA2, BRIP1, NBS1, FOXM1 and RAD51 were normalised to siCon. N=3 and error bars represent SEM. T-tests were used (p-value of: <0.05*, <0.01**, <0.001***, <0.0001****). Whole cell lysates were isolated for the breast cancer cell line (D) (Di) HCC1954, 48- and 72-hours post-transfection with si*Foxm1*(6) and si*Foxm1*(pool) were analysed on Western blot and probed with FOXM1 and RAD51 antibodies. β-tubulin was used as a loading control. N=1. Image J was used to quantify Western blots. (E) (F) HCC1954 and MCF7 were also transfected with si*Foxm1*(6) to compare siRNAs. The controls used for these transfections were siCon (negative control) and siPLK1 (positive control).

As there is already published data on FOXM1 regulating *Brip1* in breast cancer cells [117], but not for *Rad51* in breast cancer cells, we decided to focus on the FOXM1-mediated regulation of RAD51 in breast cancer. To this end, we next determined whether the change in *Rad51* mRNA expression upon *Foxm1* silencing was also translated into changes in protein expression. We immunoblotted protein lysates that had been isolated from HCC1954 cells that had been transfected with either siRNA against FOXM1 (si*Foxm1*(6) and si*Foxm1*(pool)) or a non-targeting siRNA control (siCon). Our data confirmed our RT-qPCR data, and showed a significant reduction in RAD51 protein expression, upon *Foxm1* silencing (Figure 15D). This data further corroborates a role for FOXM1 in regulating *Rad51* expression, in breast cancer cells. It also indicates that this reduction in RAD51 protein expression levels, caused by silencing *Foxm1*, may contribute to Olaparib sensitisation in Thiostrepton-treated breast cancer cells.

5.0 Inhibition of FOXM1 by Thiostrepton alters expression levels of homologous recombination proteins

5.1 Thiostrepton treatment changes expression levels of FOXM1 and homologous recombination proteins over time in breast cancer cells

To validate our results suggesting that FOXM1 regulates *Rad51* in breast cancer cells, and that this regulation may play a role in mediating Olaparib sensitisation in Thiostrepton-treated breast cancer cells, we next treated HCC1954 cells with Thiostrepton and determined changes in FOXM1 and HR gene expression over

time. We first performed RT-qPCR on HCC1954 cells that had been treated with Thiostrepton over time (Figure 16A).

50nM Thiostrepton was selected because this concentration was used for HCC1954 in Figure 12. Then, the MCF10A cell line was treated the same, with 50nM Thiostrepton, as shown in Figure 16 and Figure 17.

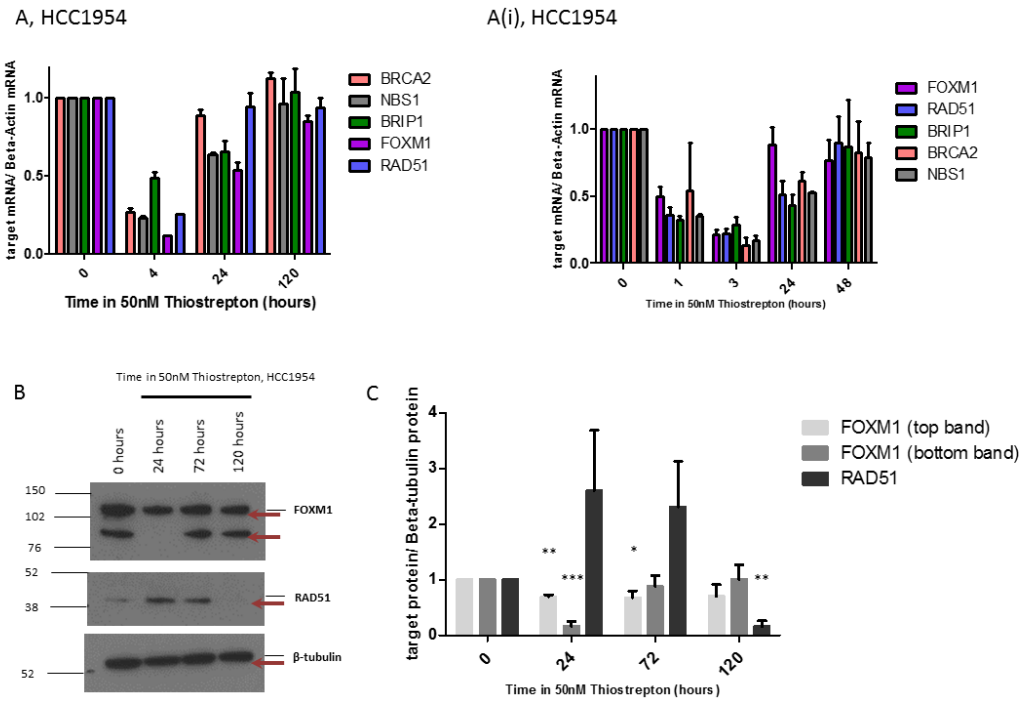


Figure 16. FOXM1 inhibition reduces expression levels of homologous recombination proteins and mRNA which is rescued over time, in breast cancer cells.

A) Ai) RNA collected at various time-points post-treatment with 50nM Thiostrepton and RT-qPCR performed. β-actin was used as a control and mRNA levels of BRCA2, BRIP1, NBS1, FOXM1 and RAD51 were normalised to vehicle control, DMSO. Two repeats are shown, at different time-points, with a similar trend. B) C) Whole cell lysates collected 24-, 72- and 120-hours post-treatment with 50nM Thiostrepton were analysed on Western blot and probed with FOXM1 and RAD51 antibodies. β-tubulin was used as a loading control. N=3 and error bars represent SEM. T-tests were used (p-value of: <0.05*, <0.01**, <0.001***).

Figure 16A shows a trend that *Foxm1* mRNA levels were reduced after 4-hours Thiostrepton treatment. In addition, we observed a corresponding decrease in the HR genes *Brac2*, *Nbs1*, *Brip1* and *Rad51*, which may be attributed to the role of FOXM1 in regulating the expression of these genes. Interestingly, *Brip1* is the least reduced HR gene at this time-point. The expression of *Brip1* may be maintained at a higher level, independently of FOXM1 regulation, so that cells can continue to repair any basal DNA damage, and any additional DNA damage caused by Thiostrepton treatment.

We also investigated the expression of *Foxm1* and HR genes at 24- and 120-hours post-treatment with Thiostrepton, to determine the effect of FOXM1 inhibition after longer exposure to the compound. After 24-hours of Thiostrepton treatment, expression levels of *Foxm1* and HR genes have recovered compared to 4-hours of Thiostrepton treatment (Figure 16A). The mechanism of action of Thiostrepton is suggested to be through binding FOXM1 and preventing the regulation of other gene promoters, but not necessarily its own promoter [203]. With this in mind, this could potentially explain why levels of *Foxm1* have increased at this time-point, because of its suggested auto-regulatory function [199]. Therefore, at 24-hour exposure to Thiostrepton, cells may be responding to the compound through up-regulating HR genes, and this could be part-mediated by the increased *Foxm1* expression levels, as well as any additional compensatory action of the cells.

By 120-hours of Thiostrepton treatment, expression levels of *Foxm1* and HR genes had recovered even more, compared to 4- and 24-hours of Thiostrepton treatment (Figure 16A). In this instance, long-term exposure to Thiostrepton may actually lead to a loss in the effect of FOXM1 inhibition on the cells. This could be what is

allowing expression levels of *Foxm1* to increase, because FOXM1 is no longer being subjected to inhibition, and this may also enhance its auto-regulatory function [199]. FOXM1 may now be regulating the HR genes to increase their expression levels, for basal DNA repair pathways.

Overall, our RT-qPCR data suggested that short-term exposure to Thiostrepton in breast cancer cells offers the most appropriate therapeutic window for our combined therapy. This is because *Foxm1* and HR gene expression levels are lower at our earliest time-point, and therefore breast cancer cells may have increased sensitisation to Olaparib after exposure to Thiostrepton. It seems that longer-term exposure to Thiostrepton causes *Foxm1* and HR expression levels to recover to higher levels that may render our combination therapy less effective.

To further investigate the effect of FOXM1 inhibition over time, we next determined how FOXM1 and RAD51 protein expression levels changed at different time-points, post-treatment with Thiostrepton. Interestingly, the most significant change in FOXM1 protein expression occurred at our earliest time-point, 24-hours post-treatment with Thiostrepton (Figure 16B-C). As with *Foxm1* mRNA data, FOXM1 protein expression is recovered over time, at 72- and more so at 120-hours post-treatment with Thiostrepton. Surprisingly, Figure 16B-C shows that Thiostrepton treatment over time caused protein expression of RAD51 to change in a different way to mRNA expression. There was an initial increase in RAD51 expression at 24-hours, and this was reduced by 120-hours, post-treatment with Thiostrepton. This could be explained by the large increase in *Rad51* mRNA expression that we observed between 4- and 24-hours treatment with Thiostrepton (Figure 16A), which seems to be subsequently translated into RAD51 protein at 24-hours treatment with

Thiostrepton (Figure 16B-C). By 120-hours exposure to Thiostrepton, breast cancer cells may be adjusting back to normal basal expression levels, which may not require high translation rates of *Rad51* mRNA. Alternatively, FOXM1 inhibition may cause a reduction in the proteins it regulates, such as RAD51, at this later time-point.

Taken together, short-term exposure to Thiostrepton also caused reduced FOXM1 protein expression, and this was increased over time, as we observed with *Foxm1* mRNA expression. As our 24-hour Thiostrepton treatment time-point showed a large increase in RAD51 protein expression, this could be based on increased *Rad51* mRNA expression between 4- and 24-hours of Thiostrepton treatment. Since the effect of FOXM1 inhibition seemed to be lost after long-term exposure to Thiostrepton, at mRNA and protein levels, the reduction in *Rad51* mRNA expression observed at 4-hours post-treatment seems a more viable therapeutic window to exploit with sensitisation to Olaparib in breast cancer cells.

5.2 Thiostrepton treatment changes expression levels of FOXM1 and homologous recombination proteins over time in non-tumourigenic breast epithelial cells

Our data so far suggested that the therapeutic window of our combination therapy would be based on short-term Thiostrepton exposure, with a subsequent sensitisation of breast cancer cells to Olaparib, through reduced expression of FOXM1 and HR proteins. We next investigated the response of non-tumourigenic breast epithelial MCF10A cells to Thiostrepton treatment over time, by analysing expression levels of FOXM1 and HR genes. Using this approach, we explored how

we could potentially increase the therapeutic window of our combination therapy, in order to spare the normal cells of breast cancer patients.

We first performed RT-qPCR on MCF10A cells that had been treated with Thiostrepton over time (Figure 17A). Interestingly, we only observed a decrease in *Foxm1* mRNA expression levels at our latest time-point of 120-hours post-treatment with Thiostrepton. This result is different to current literature, and to what we observed in HCC1954 breast cancer cells. In a previous study, Thiostrepton treatment has been shown to have no effect on protein expression levels of FOXM1 in MCF10A cells, up to 72-hours exposure [183]. In HCC1954 cells, *Foxm1* mRNA expression was reduced much earlier, at 4-hours post-treatment with Thiostrepton, and this was then recovered by 120-hours post-treatment with Thiostrepton (Figure 16A).

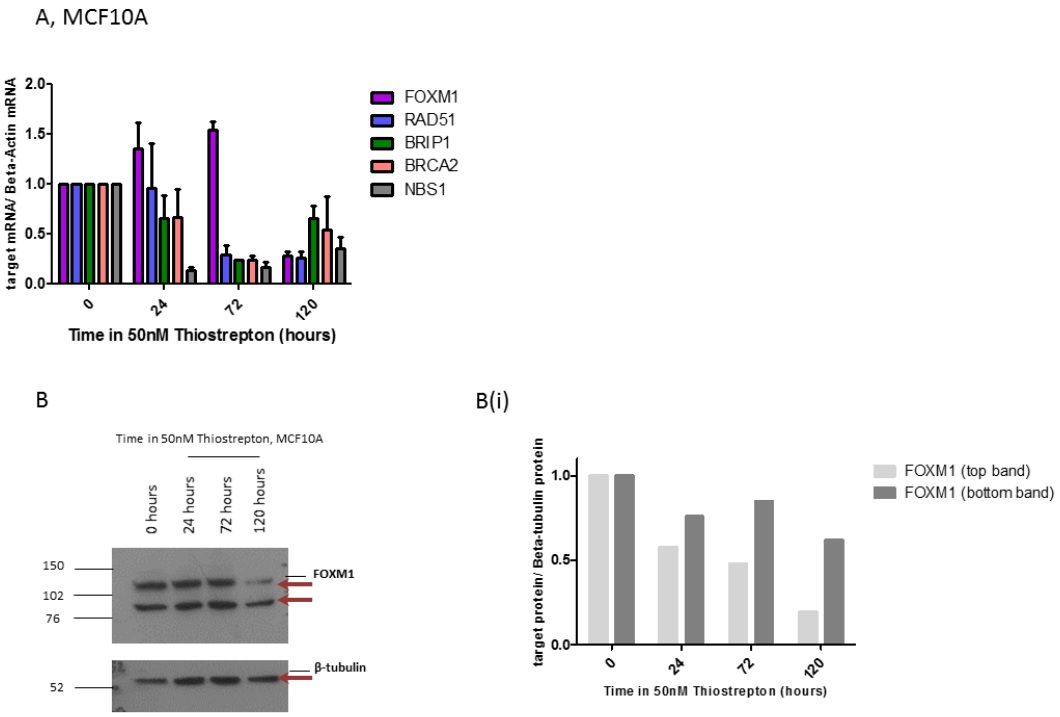


Figure 17. FOXM1 inhibition reduces expression levels of homologous recombination proteins, at later time-points in the non-tumourigenic breast epithelial cell line MCF10A, compared to breast cancer cells.

A) RNA collected 24-, 72- and 120-hours post-treatment with 50nM Thiostrepton and RT-qPCR performed. β-actin was used as a control and mRNA levels of BRCA2, BRIP1, NBS1, FOXM1 and RAD51 were normalised to vehicle control, DMSO. N=1 and error bars represent SD. B) Bi) Whole cell lysates collected 24-, 72- and 120-hours post-treatment with 50nM Thiostrepton were analysed on Western blot and probed with the FOXM1 antibody. β-tubulin was used as a loading control. N=1. Image J was used to quantify Western blots.

Our MCF10A cell line data also differed to what we observed in HCC1954 breast cancer cells, where HR genes were up-regulated over time, post-treatment with Thiostrepton (Figure 16A). FOXM1 is not the sole regulator of homologous recombination gene transcription; therefore, it could be other transcriptional regulators that have caused the observed reduction in HR gene expression before FOXM1 expression levels were reduced. This could be because the damage caused by Thiostrepton treatment was being repaired by other DNA repair pathways.

When we investigated FOXM1 protein expression levels over time in MCF10A cells treated with Thiostrepton (Figure 17B, Bi), we observed a similar trend to our mRNA data for MCF10A cells treated with Thiostrepton (Figure 17A). At both mRNA and protein level, FOXM1 expression levels were not reduced with short-term exposure to Thiostrepton. It was only after long-term exposure to Thiostrepton that we observed decreased FOXM1 expression levels. Based on this data in MCF10A cells, our therapeutic window seemed to be further supported, where short-term Thiostrepton exposure with subsequent Olaparib sensitisation could be the most effective way to treat breast cancer patients, and to spare their normal cells.

It must be noted that in Figure 16 and Figure 17, experiments were carried out in different cell lines at different times, which limits comparison. The time-points also fluctuate, which makes interpretation less conclusive and so this also limits comparison.

6.0 DNA damage is increased in cells treated with Thiostrepton and Olaprib in combination

6.1 Breast cancer cells treated in combination with Thiostrepton and Olaparib have significantly increased levels of DNA damage, compared to treatment with either agent alone

We have now shown that our combination therapy of Thiostrepton and Olaparib causes a more greatly reduced cell viability and colony formation, compared to

either agent alone, in breast cancer cells (Figure 12A-D; Figure 13A-D), and that the reduced expression levels of HR genes, upon a shorter exposure to Thiostrepton, could be involved in mediating the sensitisation of breast cancer cells to Olaparib (Figure 16A-C). We have also investigated the therapeutic window of our combination therapy, by comparing the response of breast cancer cells and non-tumourigenic breast epithelial cells to Thiostrepton treatment over time (Figure 17A-B). Our data suggested that our combination therapy may be more effective at treating breast cancer patients with a short-term exposure to Thiostrepton, so that the cells can be sensitised to Olaparib, and the patient's normal cells can be spared.

To further investigate the mechanistic basis of our combination therapy, we next determined whether levels of DNA damage differed between our treatment conditions. It has been shown previously that FOXM1-deficient cells exhibit increased DNA breaks [115, 118]. Since a number of HR genes have been shown to be under the regulation of FOXM1 in breast cancer cells [115, 117, 118], and we have also shown for the first time that *Rad51* is regulated by FOXM1 in breast cancer cells (Figure 15A-D), we decided to investigate how this role of FOXM1 may be affected in breast cancer cells treated with our combination therapy.

We therefore determined whether Thiostrepton and Olaparib in combination caused increased DNA double-strand breaks in our cells, compared to either agent alone. To this end, we treated HCC1954 cells with 50nM Thiostrepton, 5μM Olaparib, 50nM Thiostrepton + 5μM Olaparib, or vehicle control.

50nM Thiostrepton and 5µM Olaparib were selected, because these concentrations were used for HCC1954 in Figure 12.

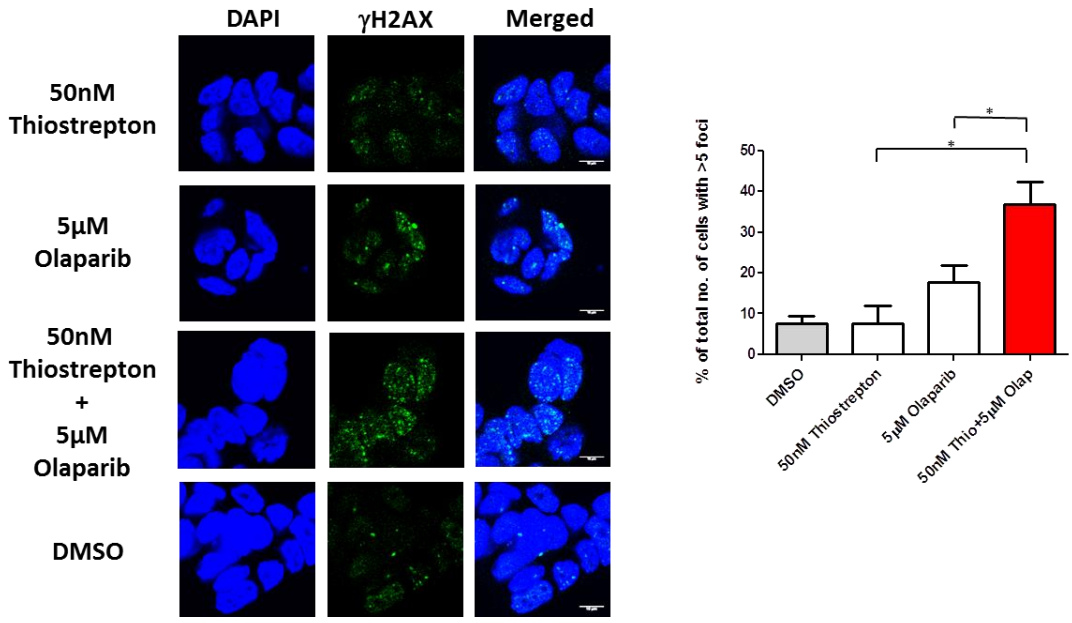


Figure 18. γH2AX foci in breast cancer cells upon treatment with Thiostrepton alone, Olaparib alone and Thiostrepton and Olaparib in combination.

Percentage of total number of cells containing more than 5 foci were measured using confocal microscopy, 24-hours post-treatment of cells on coverslips in a 24-well plate. The treatments used were 50nM Thiostrepton (white), 5µM Olaparib (white), 50nM Thiostrepton + 5µM Olaparib (red) and vehicle control, DMSO (grey). N=3 and error bars represent SEM. T-tests were used (p-value of: <0.05*).

We analysed γH2AX foci, a marker of DNA double-strand breaks, after 24-hours and determined that DNA double-strand breaks were significantly increased upon treatment with Thiostrepton and Olaparib, compared to either agent alone (Figure 18). Our results therefore suggest that increased DNA double-strand breaks may provide a mechanistic explanation for our combination therapy in breast cancer cells.

Taken together, we have shown that the role of FOXM1 in regulating DNA repair genes can be exploited therapeutically, using Thiostrepton and Olaparib in combination. Our data suggests that Olaparib sensitisation upon treatment with Thiostrepton is mediated through the HR proteins RAD51 and BRIP1, and that short-term Thiostrepton treatment may benefit patients to spare normal cells. We also showed that increased DNA DSBs may be exerting a role in the mechanism of action of our combination therapy, compared to Thiostrepton alone and Olaparib alone.

Chapter 4 - Investigating the role of FOXM1 in the oxidative stress response and its therapeutic implications

1.0 *Foxm1* silencing alters expression levels of oxidative stress pathway proteins

1.1 FOXM1 regulates the expression levels of a number of genes involved in the response to oxidative stress in breast cancer cells

Previous studies have shown that FOXM1 has been involved in a number of ways to regulate the response to oxidative stress. FOXM1 has been shown to counteract oxidative stress-induced premature senescence, by regulating Bmi-1 expression [120]. FOXM1 has also been shown to protect cells from oxidative stress by forming a negative feedback loop, stimulating the expression of antioxidant enzyme genes, and therefore counteracting increased intracellular ROS levels [119]. Using siRNA targeted against *Foxm1*, the authors demonstrated that the expression levels of the antioxidant genes MnSOD, catalase and PRDX3 were subsequently reduced [119]. They also showed that FOXM1-deficiency caused elevated ROS levels, compared to the control, further establishing a role for FOXM1 in ROS regulation [119]. In this study, we aimed to further investigate the role of FOXM1 in the oxidative stress response and whether we can exploit this therapeutically.

To identify which other genes involved in the oxidative stress response may be regulated by changes in FOXM1 expression, we performed RT-qPCR on the HCC1954, MCF7 and HS578T breast cancer cell lines that had been transfected with two different siRNAs targeting FOXM1 (si*Foxm1*(5) and si*Foxm1*(pool)) or a non-targeting siRNA control (siCon; Figure 19A-C). We measured the expression levels of a range of genes previously shown to be involved in the response to oxidative stress. These included MTCO1, NFR1, PPARGC1 and NFE2L2 (also known as NRF2). Our results suggest that upon *Foxm1* silencing, we observed a significant change in expression of *Mtco1*; one of the three cytochrome c oxidase subunit mitochondrial genes, *Nrf1*; a regulator of genes involved in oxidative stress, and *Ppargc1*; involved in mitochondrial biogenesis, but these changes were not consistent across all cell lines. However, our results did show that silencing *Foxm1* resulted in a significant decrease in *Nrf2* expression in all three breast cancer cell lines; HCC1954, MCF7 and HS578T. This validated previous work which reported that *Nrf2* is transcriptionally activated by FOXM1, and that *Foxm1* silencing causes reduced NRF2 expression, in hepatocellular carcinoma cells and bladder cancer cells [302, 303]. However, they reported that *Nrf2* silencing did not affect FOXM1 expression [302].

To determine whether this change in *Nrf2* mRNA expression was also translated into changes in protein expression, we immunoblotted protein lysates that had been isolated from HCC1954, MCF7 and HS578T breast cancer cell lines that had been transfected with either siRNA against FOXM1 (si*Foxm1*(5) and si*Foxm1*(pool)) or a non-targeting siRNA control (siCon). As the NRF2-KEAP1 cytoplasmic interaction is regulated by protein kinase C phosphorylation, to enable NRF2 nuclear translocation in response to oxidative stress [313, 314], we analysed the expression of both phosphorylated NRF2 and total NRF2 (Figure 19D-E), upon *Foxm1*

silencing. Our data showed that phosphorylated NRF2 and total NRF2 expression levels were decreased, confirming our RT-qPCR data and suggesting that both forms of NRF2 are affected by *Foxm1* silencing. The consequence of decreased phosphorylated NRF2 and total NRF2 could lead to a reduced activity of the antioxidant response. This may also result in additional effects on proteins downstream of NRF2. We next investigated NQO1, an enzyme that is regulated by NRF2, through binding to the antioxidant response element (ARE) consensus sequence in NQO1 [315]. Roles of NQO1 include the reduction of quinones to hydroquinones, by-passing the generation of highly reactive semiquinones [316]. This role has been exploited by using bioactive drugs, such as β-lapachone, that require NQO1 activity, and subsequently leads to harmful cellular events including ROS formation, DNA damage and apoptosis, in pancreatic cancer cells as well as head and neck cancer cells [317, 318]. Other roles include the direct scavenging of reactive oxygen species [141]. Surprisingly, we observed an increase in NQO1 expression, upon *Foxm1* silencing in the HCC1954 and MCF7 breast cancer cell lines (Figure 19E). As we observed that *Foxm1* silencing reduced NRF2 expression, it could be expected that *Foxm1* silencing would also cause a corresponding reduction in the expression of NQO1. Therefore, this increased NQO1 expression may be a compensatory action of the cells to *Foxm1* silencing, and this may not be mediated by the NRF2 pathway.

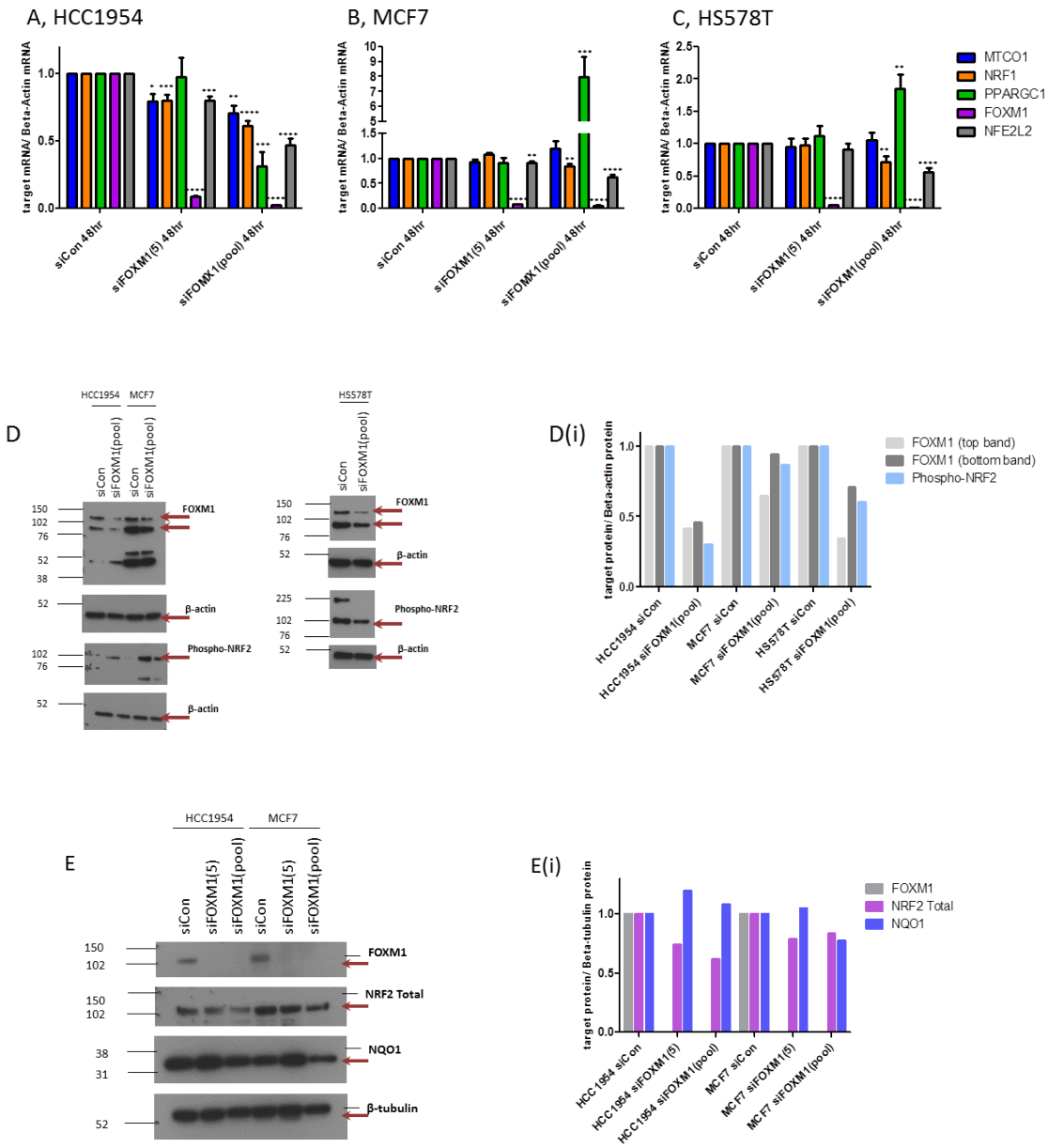


Figure 19. FOXM1 regulates the expression levels of a number of genes involved in the response to oxidative stress in breast cancer cells.

A) B) C) RNA collected 48-hours post-transfection with si*Foxm1*(5) and si*Foxm1*(pool) and RT-qPCR performed. β-actin was used as a control and mRNA levels of MTCO1, NRF1, PPARGC1, FOXM1 and NFE2L2 were normalised to siCon. N=3 and error bars represent SEM. T-tests were used (p-value of: <0.05*, <0.01**, <0.001***, <0.0001****). D) Di) E) Ei) Whole cell lysates collected 72-hours post-transfection with si*Foxm1*(5) and si*Foxm1*(pool) were analysed on Western blot and probed with FOXM1, phospho-NRF2, NRF2 total and NQO1 antibodies. β-actin and -tubulin were used as loading controls. N=1. Image J was used to quantify Western blots. The controls used for these transfections were siCon (negative control) and siPLK1 (positive control).

2.0 Chemotherapy resistance causes upregulation of FOXM1 and the oxidative stress protein NQO1

2.1 FOXM1 and NQO1 have increased expression levels in platinum-resistant ovarian cancer cells

Our data thus far suggested that FOXM1 can regulate NRF2 and NQO1 expression. Previous studies have shown evidence that all three of these proteins play a role in chemoresistance [188, 319, 320]. Therefore, we next aimed to explore this further in our study. FOXM1 has been suggested to mediate acquired Cisplatin resistance in breast cancer cells, through a proposed mechanism involving the DNA repair pathway [188]. Other studies have linked FOXM1 to chemoresistance in ovarian cancer [174, 190], lung cancer [321], and there have also been additional studies in breast cancer [191, 322]. Another study investigated NRF2, and subsequently NQO1, in lung carcinoma, breast adenocarcinoma and neuroblastoma, and suggested that these proteins were involved in mediating chemoresistance [319]. Other studies have linked NRF2 to chemoresistance in pancreatic cancer [323], and NQO1 to chemoresistance in cholangiocarcinoma [320].

Since FOXM1, NRF2, NQO1 expression levels have been implicated in chemoresistance in different cancer types, we used an established model of ovarian cancer cells that are chemosensitive and chemoresistant to further investigate these proteins in our chosen model. The parental ovarian cancer cell line Ovcar4, the Cisplatin-resistant ovarian cancer cell line Ov4cis and the Carboplatin-resistant cell line Ov4carbo were kindly provided to us by the Lockley lab, BCI.

We first validated chemotherapy sensitivity in our cell lines. We treated our cells with increasing concentrations of Cisplatin (Figure 20A) or increasing concentrations of Carboplatin (Figure 20B) and analysed cell viability after 72-hours. A range of chemotherapy concentrations were tested in order to observe differences in sensitivity between chemo-sensitive and -resistant ovarian cancer cell lines. The IC50 of Cisplatin for Ovcar4 and Ov4cis were around 3µM and around 16µM, respectively (Lockley lab, BCI, personal communication). Therefore, we selected concentrations either side of these values. Our data confirmed that the Ov4cis cells were resistant to Cisplatin (IC50 14.9µM, Table 7), compared to Ovcar4 (IC50 3.0µM, Table 7), and the Ov4carbo cells were resistant to Carboplatin (IC50 out of range, Table 7), compared to Ovcar4 (IC50 44.4µM, Table 7), as expected.

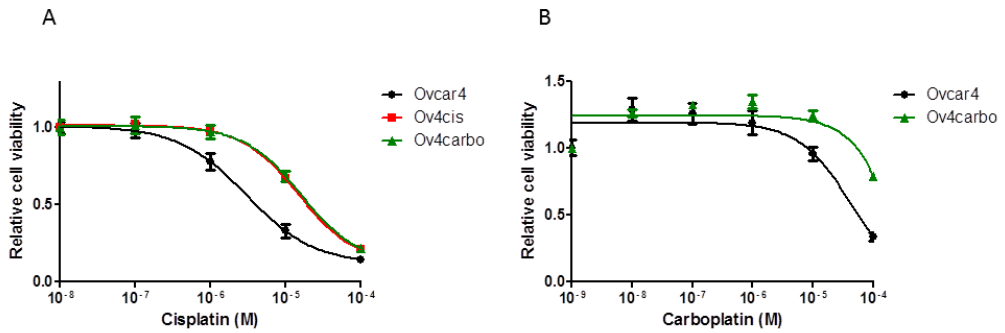


Figure 20. Validating chemotherapy sensitivity in our ovarian cancer cells.

A) Dose response curves for Cisplatin in ovarian cancer cells, after 72-hours treatment in a 96-well plate. N=2 and error bars represent SE. B) Dose response curves for Carboplatin in ovarian cancer cells, after 72-hours treatment in a 96-well plate. N=1 and error bars represent SD.

Table 7. IC50 values for ovarian cancer cell lines treated with Cisplatin and Carboplatin.

Cell line, compound	IC50 value (µM)
Ovcar4, Cisplatin	3.0
Ov4cis, Cisplatin	14.9
Ov4carbo, Cisplatin	16.2
Ovcar4, Carboplatin	44.4
Ov4carbo, Carboplatin	Out of range

Once our cell lines had been validated, we next immunoblotted protein lysates that had been isolated from Ovcar4, Ov4cis and Ov4carbo ovarian cancer cell lines for basal protein expression levels of FOXM1 and NQO1. Since NRF2 is upstream of NQO1, and FOXM1 directly regulates NRF2 expression, we decided to focus the rest of our study on FOXM1, and a downstream protein, NQO1, and their roles in chemoresistance. Our results showed that FOXM1 expression levels were increased in platinum-resistant ovarian cancer cells, compared to parental ovarian cancer cells (Figure 21A-B).

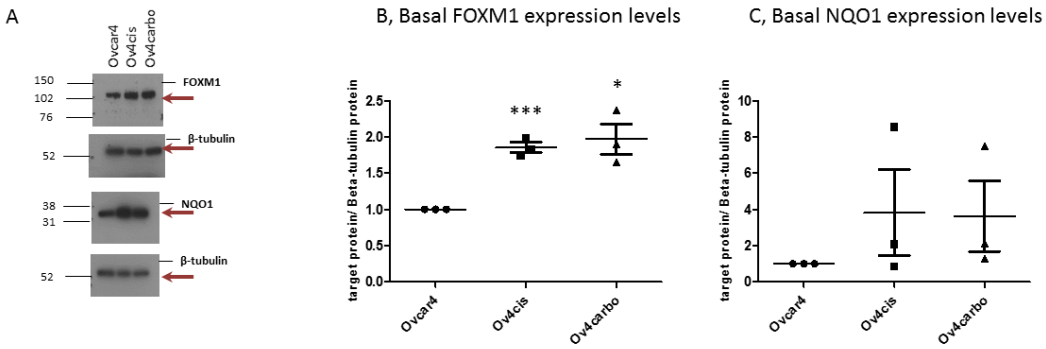


Figure 21. FOXM1 expression levels are increased in platinum-resistant ovarian cancer cells.

A) Whole cell lysates were analysed on a Western blot and probed with FOXM1 and NQO1 antibodies. β-tubulin was used as a loading control. B) C) FOXM1 and NQO1 blots were quantified. ImageJ analysis was used, and normalisation was to the

parental cell line Ovcar4. N=3 and error bars represent SEM. T-tests were used (p-value of: <0.05*, <0.001***).

This confirmed published data that FOXM1 is upregulated in chemoresistant cancer cells. We now have a model to further investigate the mechanistic and therapeutic implications of targeting FOXM1 and the oxidative stress response pathway in cancer cells. We next wanted to determine if these proteins play roles separately, or together, in driving cancer cells to chemoresistance.

3.0 Investigating chemoresistance in ovarian cancer cells, and whether this is mediated by FOXM1 and NQO1

3.1 *Foxm1* silencing results in increased NQO1 expression, and *Nqo1* silencing results in decreased FOXM1 expression

Our data suggests that upon *Foxm1* silencing in breast cancer cells, NQO1 expression is increased (Figure 19E). To determine whether this data was a specific feature of breast cancer cells, we immunoblotted protein lysates that had been isolated from the Ovcar4, Ov4cis and Ov4carbo ovarian cancer cell lines that had been transfected with either siRNA against FOXM1 (si*Foxm1*(5)) or a non-targeting siRNA control (siCon). We subsequently analysed the expression of NQO1 (Figure 22A, C) upon *Foxm1* silencing. As observed in breast cancer cells (Figure 19E), we also observed an increase in NQO1 expression when *Foxm1* had been silenced. To investigate this further, we transfected the ovarian cancer cells with either siRNA

against NQO1 (si*Nqo1*(pool)) or a non-targeting siRNA control (siCon) and after 72-hours isolated protein lysates. Significantly, we observed a decrease in FOXM1 expression upon *Nqo1* silencing (Figure 22A-B).

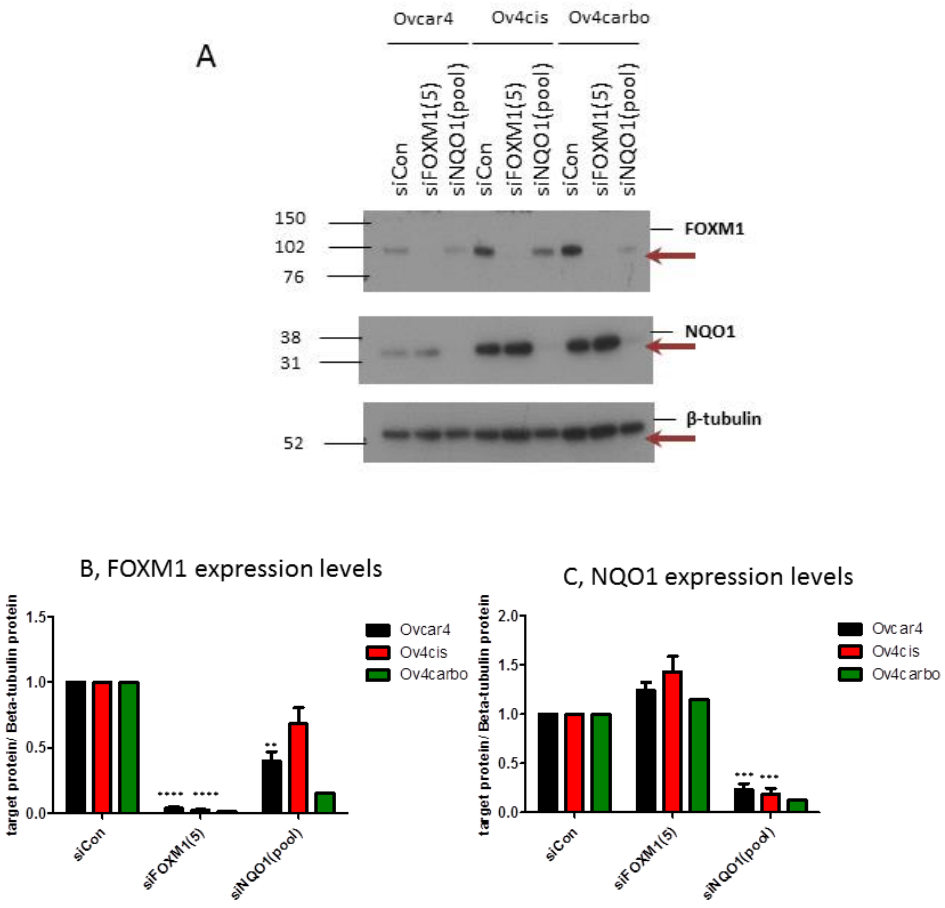


Figure 22. Foxm1 silencing results in increased NQO1 expression and Nqo1 silencing results in decreased FOXM1 expression, in ovarian cancer cells.

A) Whole cell lysates collected 48-hours post-transfection with si*Foxm1*(5) and si*Nqo1*(pool) were analysed on a Western blot and probed with FOXM1 and NQO1 antibodies. β-tubulin was used as a loading control. The controls used for this transfection were siCon (negative control) and siPLK1 (positive control). B) C) FOXM1 and NQO1 blots were quantified. ImageJ analysis was used, and normalisation was to siCon. N=3 (Ovcar4 and Ov4cis), N=1 (Ov4carbo), and error bars represent SEM. T-tests were used (p-value of: <0.01**, <0.001***, <0.0001****).

These data suggest that FOXM1 and NQO1 expression are potentially regulated by each other and may have compensatory functional roles in the cell.

si*Foxm1*(5) only was tested in ovarian cancer cell lines, and it showed the greatest, reproducible knock-down of *Foxm1* across multiple experiments. Another siRNA targeted to *Foxm1*, as well as another siRNA targeted to *Nqo1*, will also need to be tested to validate data with si*Foxm1*(5) and si*Nqo1*(pool).

It must also be noted that there is a caveat to this experiment, and the following experiments, where using a single siRNA targeted to *Foxm1*/*Nqo1* does not allow full interpretation. Therefore, future experiments should test a second siRNA targeted to *Foxm1*, and a second siRNA targeted to *Nqo1*.

3.2 Thiostrepton treatment significantly increases expression levels of NQO1

After having observed increased expression of NQO1 upon *Foxm1* silencing in breast cancer cells and ovarian cancer cells (Figure 19E; Figure 22A, C), we next used the FOXM1 inhibitor Thiostrepton as another means to inhibit FOXM1 activity and to determine its effect of NQO1 expression. At this stage, we also investigated the expression of another downstream enzyme of the NRF2 pathway, SOD1, which is involved in catalysis of the potentially toxic superoxide to a less toxic substance hydrogen peroxide. SOD1 is a superoxide dismutase, and in mammals there are three SOD isoforms (the cytoplasmic Cu/ZnSOD (SOD1), the mitochondrial MnSOD (SOD2), and the extracellular Cu/ZnSOD (SOD3)) [324]. Interestingly, MnSOD (SOD2) expression levels have already been shown to be reduced upon *Foxm1* silencing [119].

We treated Ovcar4, Ov4cis and Ov4carbo cells with either a vehicle control (DMSO), 1μM Thiostrepton or 2μM Thiostrepton, and isolated total protein lysates. Our results suggested that inhibition of FOXM1 increased expression of NQO1 (Figure 23A, C), as was observed upon siRNA-mediated *Foxm1* silencing (Figure 19E; Figure 22A, C).

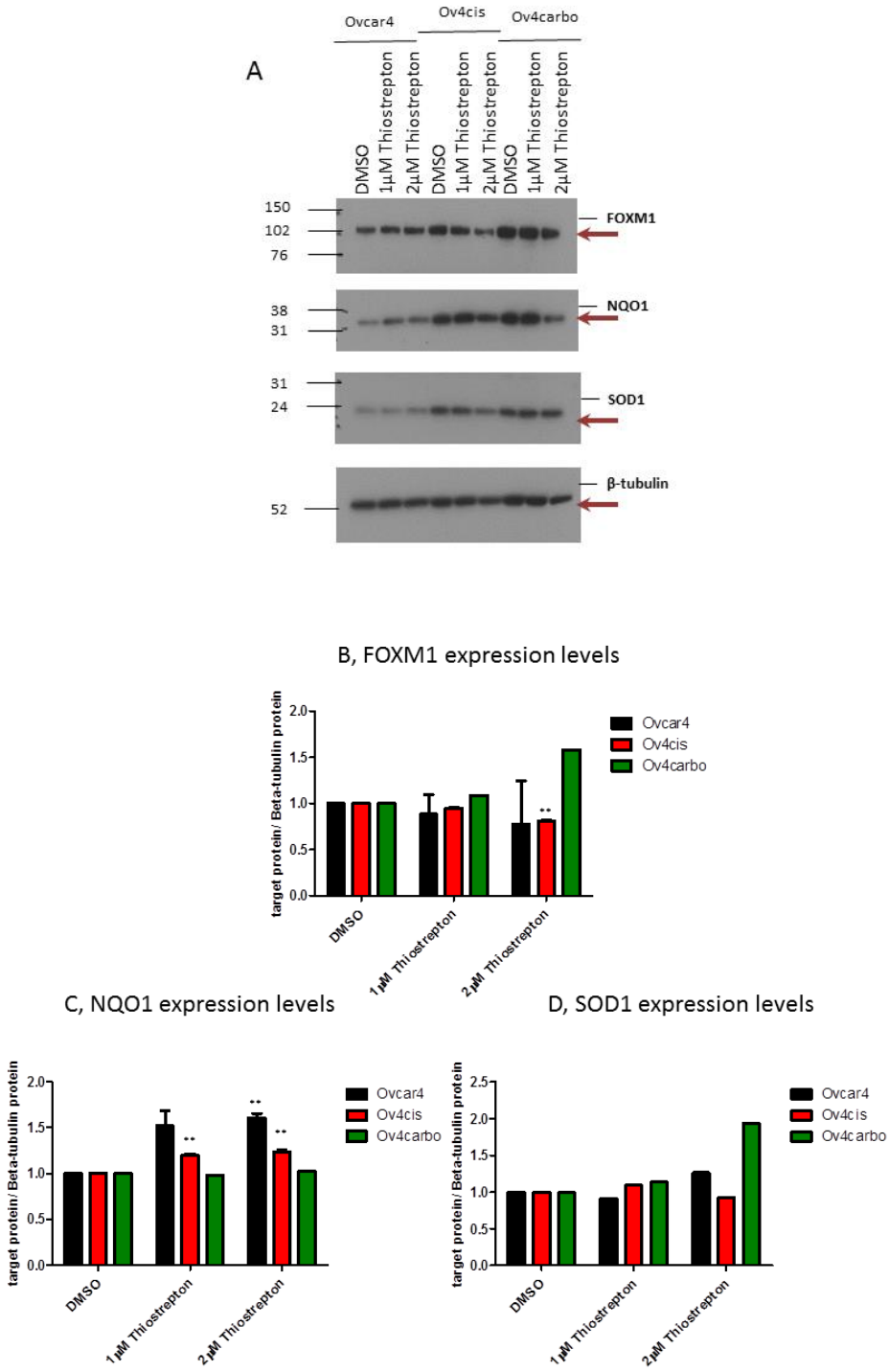


Figure 23. FOXM1 inhibition does affect protein expression levels of the oxidative stress proteins NQO1 and SOD1, in ovarian cancer cells.

A) Whole cell lysates collected 48-hours post-treatment with 1μM Thiostrepton, 2μM Thiostrepton or vehicle control were analysed on a Western blot and probed with FOXM1, NQO1 and SOD1 antibodies. β-tubulin was used as a loading control. B) C) D) FOXM1, NQO1 and SOD1 blots were quantified. ImageJ analysis was used, and normalisation was to vehicle control sample, DMSO. N=2 (Ovcar4 and Ov4cis for FOXM1 and NQO1), N=1 (Ov4carbo for FOXM1 and NQO1), N=1 (Ovcar4,

Ov4cis and Ov4carbo for SOD1), and error bars represent SEM. T-tests were used (p-value of: <0.01**).

Furthermore, Thiostrepton is an allosteric regulator of FOXM1 and prevents the interaction of the transcription factor FOXM1 with several gene promoters, but not necessarily to its own [203]. While siRNA-mediated silencing is through mRNA degradation. This difference may explain why FOXM1 expression levels are not always decreased upon treatment with Thiostrepton. Interestingly, we observed increased expression of SOD1 in the chemotherapy-resistant cell lines, independent of siRNA transfection, as we have observed for FOXM1 and NQO1.

3.3 Cisplatin treatment significantly increases expression levels of FOXM1 and NQO1

Our data suggests that FOXM1 and NQO1 expression is increased in platinum-resistant ovarian cancer cells (Figure 21A-B). Previously, Cisplatin has been shown to induce FOXM1 expression in ovarian cancer cells [308]. Furthermore, kidney adenocarcinoma cells treated with Cisplatin were shown to have increased ROS production and increased NQO1 expression [325]. Therefore, we next investigated whether short-term treatment with Cisplatin could regulate the expression levels of FOXM1 and NQO1 and whether increasing concentrations of Cisplatin caused a parallel increase in FOXM1 and NQO1 expression, in our cells. To this end, we immunoblotted protein lysates that had been isolated from Ovcar4 and Ov4cis ovarian cancer cell lines treated with either 1μM Cisplatin, 2μM Cisplatin, 3μM Cisplatin, 4μM Cisplatin or a vehicle control for 24-hours. Our data suggested that FOXM1 and NQO1 expression was increased upon Cisplatin treatment, but this was not dependent of the chemoresistance of the cells (Figure 24A-B).

Cisplatin concentrations were used based on Figure 20 to ensure low toxicity, where increasing concentrations were tested to determine whether this caused changes in FOXM1 and or NQO1 expression levels.

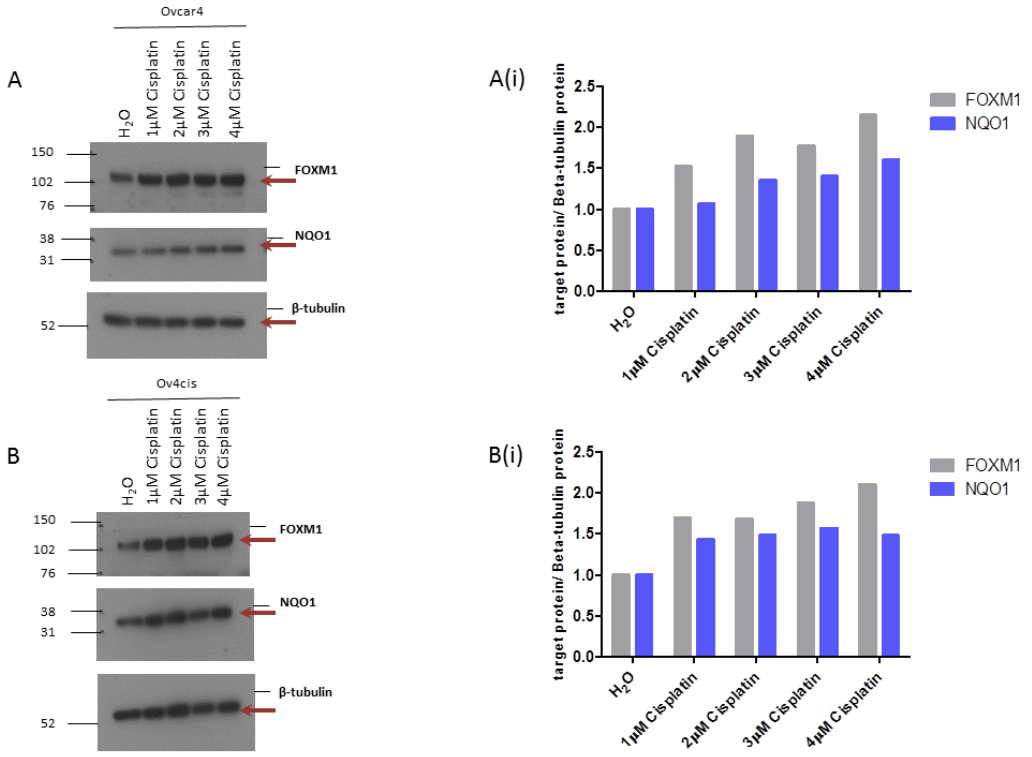


Figure 24. Cisplatin treatment significantly increases protein expression levels of FOXM1 and NQO1 in ovarian cancer cells.

A) Ai) B) Bi) Whole cell lysates collected 24-hours post-treatment with 1μM Cisplatin, 2μM Cisplatin, 3μM Cisplatin, 4μM Cisplatin or vehicle control were analysed on a Western blot and probed with FOXM1 and NQO1 antibodies. β-tubulin was used as a loading control. N=1. Image J was used to quantify Western blots.

This result therefore suggests that FOXM1 and NQO1 may play a role in generating chemoresistance to Cisplatin in ovarian cancer cells.

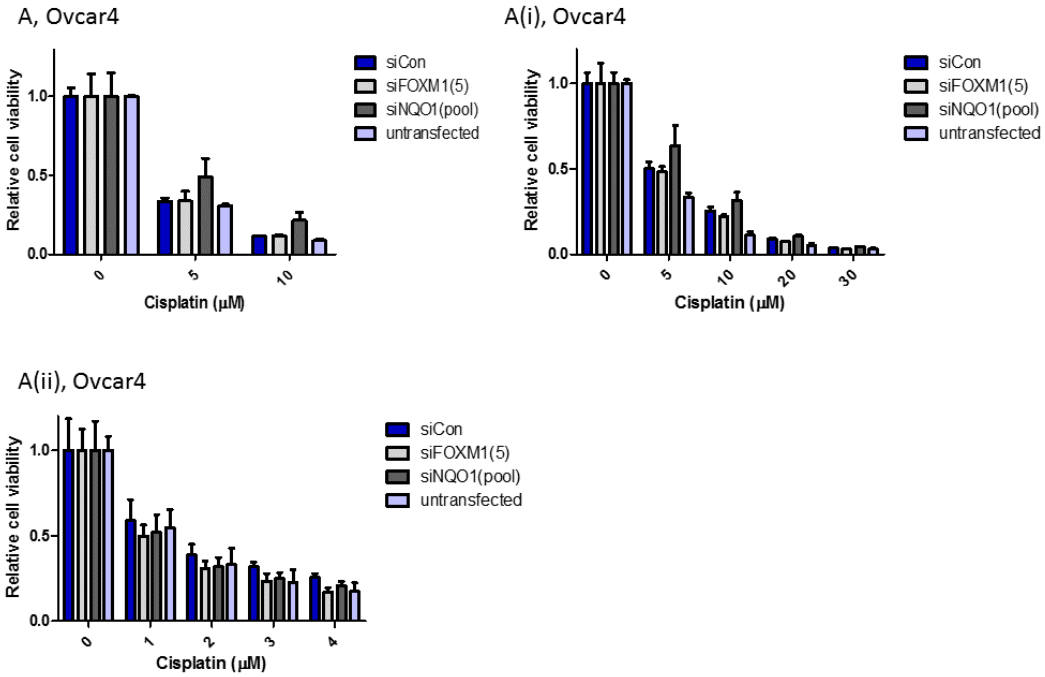
3.4 Cisplatin-resistant cells are not re-sensitised to Cisplatin upon *Foxm1* or *Nqo1* silencing

Our data suggests a relationship between the proteins FOXM1 and NQO1, and chemoresistance in platinum-resistant ovarian cancer cells, as we observed increased expression of both proteins in platinum-resistant cells (Figure 21A-B), and upon short-term Cisplatin treatment (Figure 24A-B). We have also observed that when FOXM1 or NQO1 are individually silenced, this caused a corresponding change in the expression of the other protein (Figure 22A-C). We therefore next determined the role FOXM1 and NQO1, alone or together, in the re-sensitisation of platinum-resistant cells to Cisplatin. To this end, we transfected with either siRNA against FOXM1 (si*Foxm1*(5)), siRNA against NQO1 (si*Nqo1*(pool)) or a non-targeting siRNA control (siCon) for 24-hours, and this was followed by treating the cells with either 5μM Cisplatin or 10μM Cisplatin or vehicle control. We analysed cell viability after 120-hours Cisplatin treatment. Our results suggest that in the Ov4cis cells, there was no re-sensitisation to Cisplatin with *Foxm1* silencing or *Nqo1* silencing alone at the concentrations tested (Figure 25B). Interestingly, there was a trend for increased resistance to Cisplatin when *Nqo1* was silenced in the parental Ovcar4 cells (Figure 25A), which was not observed in the Ov4cis cells. Previously, we observed that FOXM1 expression was decreased upon *Nqo1* silencing (Figure 22A-B) therefore this resistance to Cisplatin in NQO1-depleted cells may be due to the combined reduction in FOXM1 and NQO1 expression. Therefore, we hypothesise that NQO1 and FOXM1 may play a role together in causing chemoresistance in Ovcar4 cells.

When *Foxm1* was silenced, there were no changes to the trend in cell viability in chemoresistant Ov4cis cells (Figure 25B). This result indicates that FOXM1 does

not appear to play a role in resistance in ovarian cancer. The same can be said for NQO1, because *Nqo1* was silenced, there were no changes to the trend in cell viability in Ov4cis cells (Figure 25B).

Cisplatin concentrations were used based on Figure 20 to ensure low toxicity, where increasing concentrations were tested to determine whether this caused changes in cell viability when *Foxm1* or *Nqo1* were silenced. This also applied to Figure 28, when *Foxm1* and *Nqo1* were silenced together.



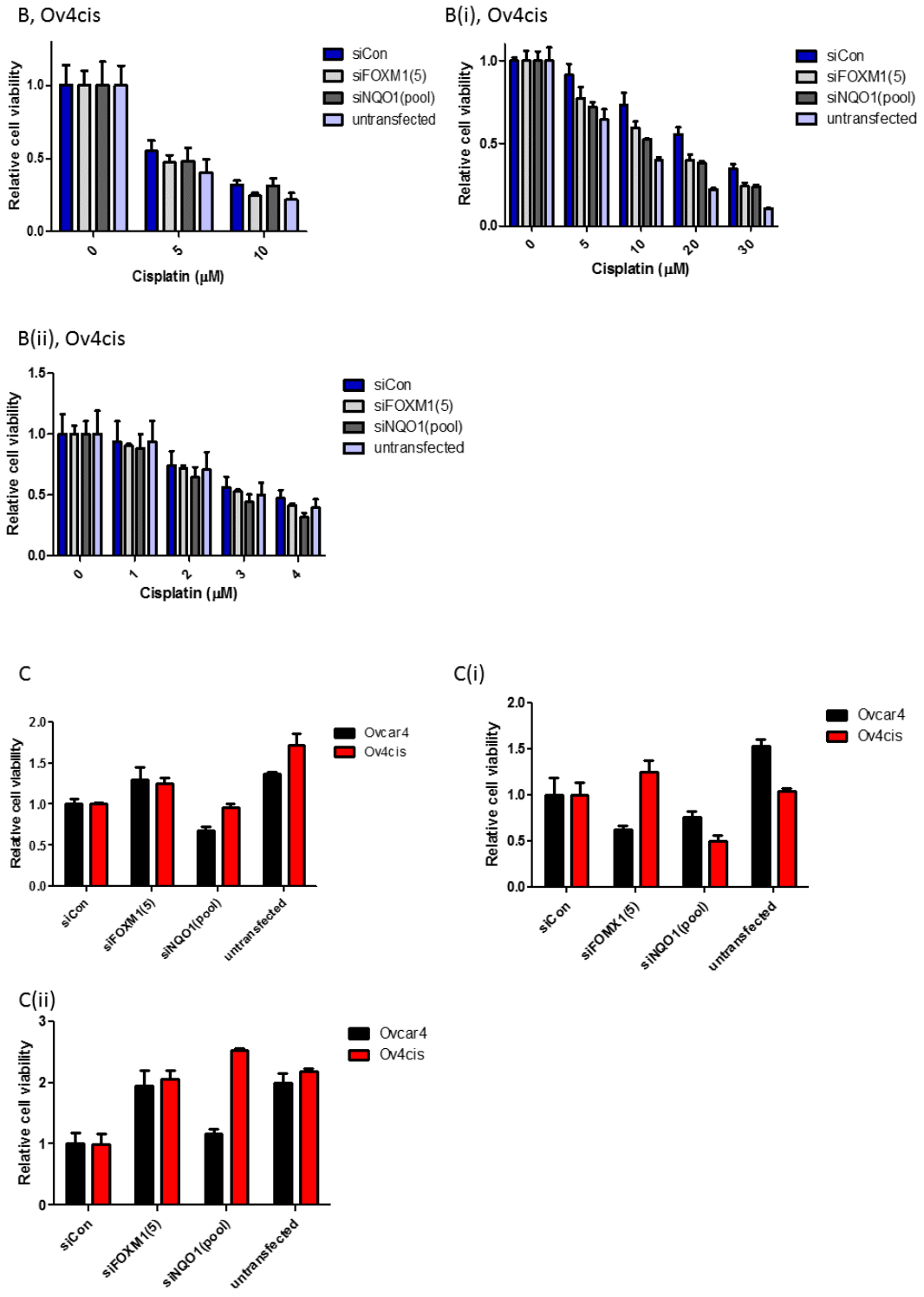


Figure 25. Cisplatin-resistant cells are not re-sensitised to Cisplatin upon *Foxm1* or *Nqo1* silencing.

A) Ai, ii) B) Bi, ii) Cisplatin sensitisation was measured using CTG, post-treatment with Cisplatin, after *Foxm1* silencing or *Nqo1* silencing in a 96-well plate. Three experiments shown for each cell line. C) Ci, ii) Cell viability was also measured without drug treatment using CTG, post-transfection with si*Foxm1*(5) or si*Nqo1*(pool) in a 96-well plate. Three experiments shown for both cell lines. The

controls used for this transfection were siCon (negative control) and siPLK1 (positive control), data not shown for siPLK1.

Furthermore, the reduction in cell viability upon silencing of *Nqo1* was greater in Ovcar4 cells (Figure 25C), and interestingly these cells have lower expression of NQO1, in comparison to the platinum-resistant cells. Therefore, perhaps Ovcar4 cells have a threshold of expression of NQO1 required for viability, and loss of NQO1 results in a reduction in cell viability, independent of Cisplatin treatment.

4.0 Do FOXM1 and NQO1 act together to mediate resistance in Cisplatin-resistant ovarian cancer cells?

4.1 *Foxm1* + *Nqo1* silencing causes reduced cell viability compared to alone in ovarian cancer cells

Our data suggests that FOXM1 and NQO1 individually do not mediate resistance to Cisplatin in Ovcar4 cells, but may act together in these cells to cause chemotherapy-resistance. To investigate this further, we silenced *Foxm1* and *Nqo1* alone or together. We determined the effect on cell viability of silencing *Foxm1* and *Nqo1* alone or together, by transfecting Ovcar4, Ov4cis and Ov4carbo cells with either siRNA against FOXM1 (si*Foxm1*(5)), siRNA against NQO1 (si*Nqo1*(pool)) or together and a non-targeting siRNA control (siCon) for 144-hours and then measured cell viability. Our results suggest that loss of both FOXM1 and NQO1 together resulted in a trend for reduced cell viability in all cell lines, in comparison to silencing either FOXM1 or NQO1 alone (Figure 26). These experiments were controlled for the amount of siRNA used to ensure this increased toxicity upon loss

of FOXM1 and NQO1 together was not due to an increase in siRNA concentration (Materials and Methods, Section 4.2.2).

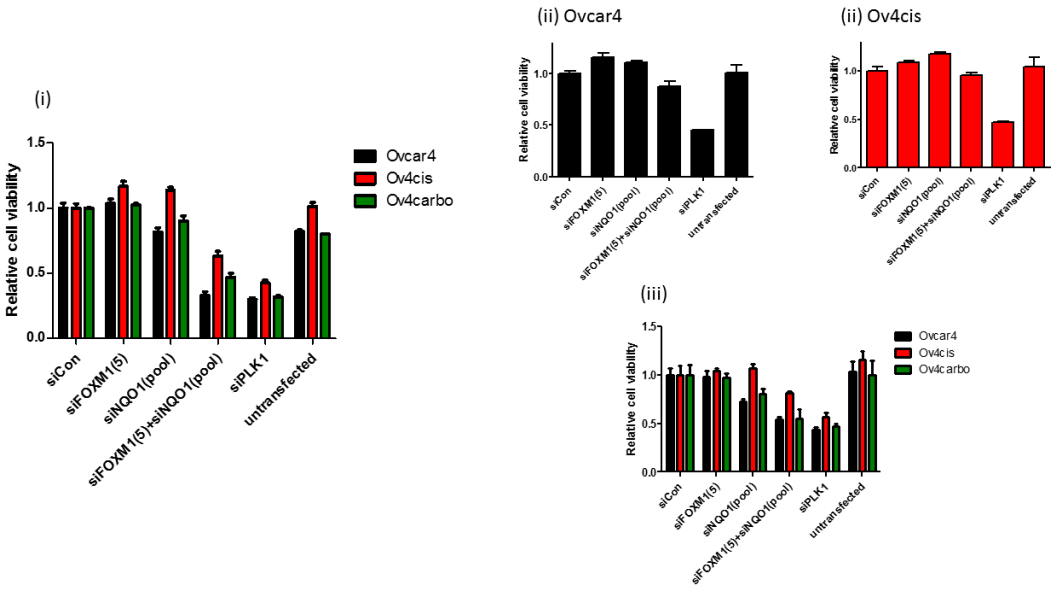


Figure 26. Cell viability without drug treatment upon Foxm1 silencing and Nqo1 silencing, alone or together, in ovarian cancer cells.

Cell viability was measured using CTG, post-transfection with si*Foxm1*(5) and si*Nqo1*(pool), alone or together, in a 96-well plate. The controls used for this transfection were siCon (negative control) and siPLK1 (positive control). Repeats are displayed, and error bars represent SD.

The reduction in cell viability upon silencing of *Foxm1* and *Nqo1* together was also greater in Ovcar4 cells. These cells have lower expression of both NQO1 and FOXM1, in comparison to the platinum-resistant cells, so Ovcar4 cells may have a threshold of expression of both FOXM1 and NQO1 required for viability, and loss of both results in a reduction in cell viability.

4.2 *Foxm1* + *Nqo1* silencing causes additional reduced colony formation compared to alone in ovarian cancer cells

To validate our results suggesting that loss of both FOXM1 and NQO1 causes a greater reduction in cell viability, compared to either alone, we also analysed loss of both proteins by clonogenic assay. To this end, we transfected with either siRNA against FOXM1 (si*Foxm1*(5)), siRNA against NQO1 (si*Nqo1*(pool)), siRNA against FOXM1 (si*Foxm1*(5)) + siRNA against NQO1 (si*Nqo1*(pool)) or a non-targeting siRNA control (siCon) for 6 days, when we subsequently stained, dissolved and measured absorbencies of remaining colonies on day 8. Significantly, we also observed a reduction in colony formation upon depletion of *Foxm1* and *Nqo1* together, in comparison to either gene alone, across all cell lines (Figure 27A-C).

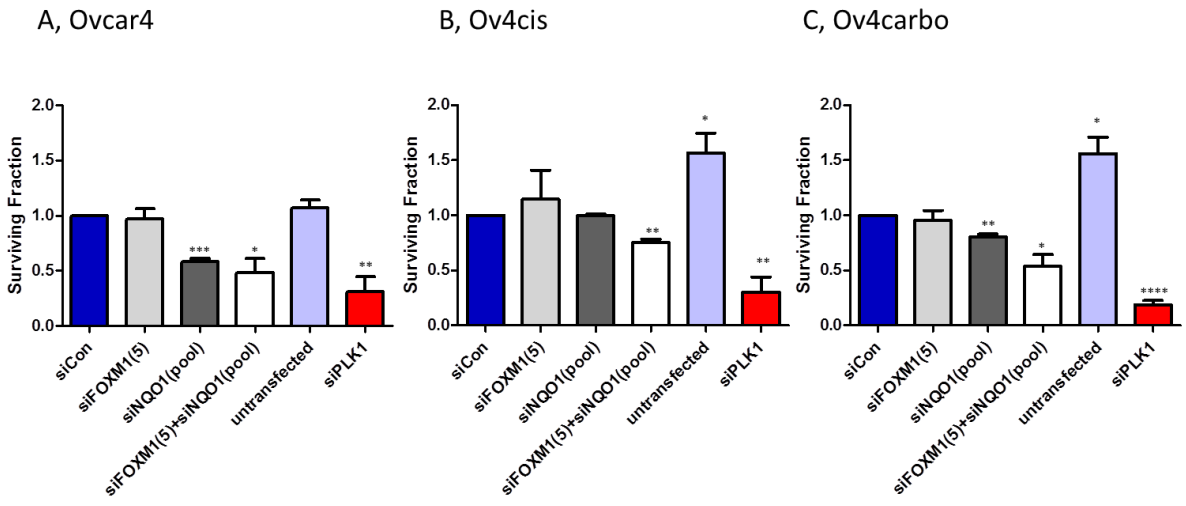


Figure 27. Colony formation without drug treatment upon Foxm1 silencing and Nqo1 silencing, alone or together, in the ovarian cancer cells.

A) B) C) Colony formation was measured using SRB staining and dissolving colonies, on day 8 post-transfection with si*Foxm1*(5) and si*Nqo1*(pool), alone (grey) or together (white), in a 6-well plate. The controls used for this transfection were siCon (negative control) (blue) and siPLK1 (positive control) (red), and data was

normalised to siCon. N=3 and error bars represent SEM. T-tests were used (p-value of: <0.05*, <0.01**, <0.001***, <0.0001****).

This effect was also the greatest in Ovcar4 cells compared to the platinum-resistant cells, so this confirmed our cell viability data and further suggested that Ovcar4 cells may have a threshold of expression of both FOXM1 and NQO1 required for viability, and loss of both results in a reduction in cell viability and colony formation.

4.3 Cisplatin-resistant cells are not re-sensitised to Cisplatin upon *Foxm1 + Nqo1* silencing

Our data thus far suggests that loss of both FOXM1 and NQO1 causes a reduction in cell viability (Figure 26) and colony formation (Figure 27A-C). We next wanted to determine whether this loss of expression could re-sensitise platinum-resistant cells to Cisplatin. We transfected the Ovcar4 and Ov4cis cells with either siRNA against FOXM1 and NQO1, alone or together, or a non-targeting siRNA control (siCon), followed by treating the cells with increasing concentrations of Cisplatin, or vehicle control. We analysed cell viability after 96-hours post-treatment with Cisplatin. Figure 28B shows that there was no re-sensitisation to Cisplatin with *Foxm1* silencing + *Nqo1* silencing at the concentrations tested, as observed for single transfections in Figure 25B. Consistent with data in Figure 25A, there was increased resistance to Cisplatin when *Nqo1* was silenced alone in the Ovcar4 cells, and this was not observed in the Ov4cis cells.

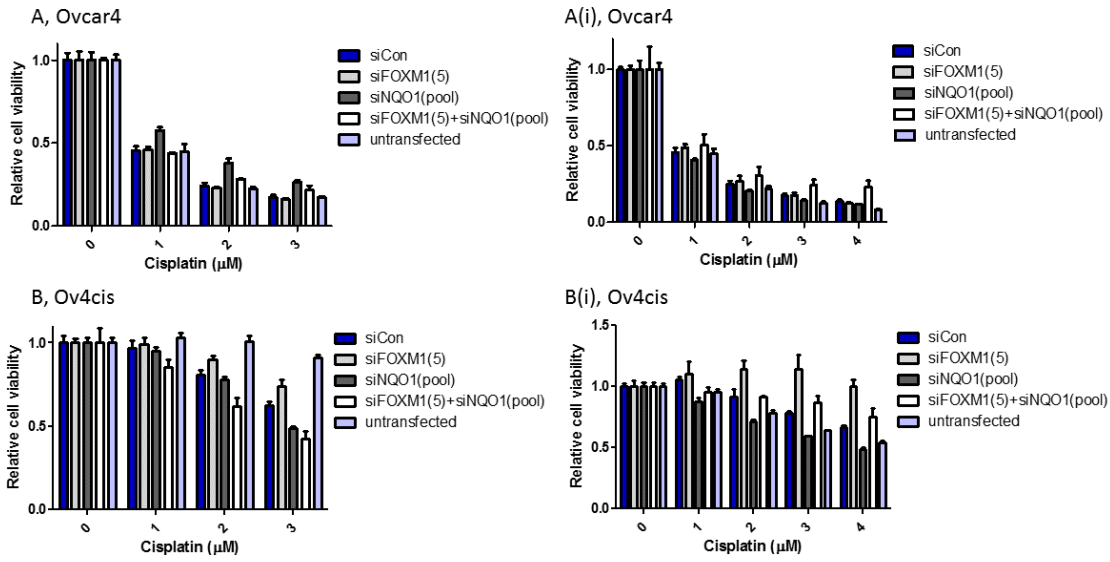


Figure 28. Cisplatin-resistant cells are not re-sensitised to Cisplatin upon Foxm1 + Nqo1 silencing.

A) Ai) B) Bi) Cisplatin sensitisation was measured using CTG, post-treatment with Cisplatin after *Foxm1* silencing and *Nqo1* silencing, alone or together, in a 96-well plate. The controls used for this transfection were siCon (negative control) and siPLK1 (positive control), and data was normalised to siCon. Two repeats are shown for each cell line, and error bars represent SD.

Ov4cis cells had a reduced cell viability when Nqo1 alone was silenced, and this appears to be the opposite of Ovcar4 cells, however it must also be noted that there was toxicity observed for siCon in Ov4cis cells (Figure 28). Therefore, it would be difficult to conclude that this effect is the opposite of Ovcar4 cells without optimising the experiment to ensure that there was no experimental toxicity due to siRNA transfection.

4.4 The FOXM1 inhibitor Thiostrepton sensitises ovarian cancer cells to Cisplatin

To further understand the role of FOXM1 and NQO1 in Cisplatin resistance, we next used the FOXM1 inhibitor Thiostrepton to see whether an alternate means of

inhibiting FOXM1 may influence the sensitivity to Cisplatin. To this end, we transfected with either siRNA against NQO1 or a non-targeting siRNA control (siCon) and treated the cells with 1μM Thiostrepton +/- 3μM Cisplatin or vehicle control. We analysed cell viability after 96-hours drug treatment. Cisplatin concentrations were again based on previous experiments in this project, and Thiostrepton concentrations were based on literature in ovarian cancer cells to ensure FOXM1 inhibition and low toxicity [173]. This also supported Thiostrepton concentrations used in Figure 23.

Our previous data was confirmed, where *Nqo1* silencing caused resistance to Cisplatin in the Ovcar4 cells. Treatment with Thiostrepton confirmed previously published data, and Thiostrepton caused sensitisation to Cisplatin in Ovcar4 and Ov4cis cells, compared to treatment with either agent alone (Figure 29A-B) [188, 308, 309]. Data was not combined because of Thiostrepton toxicity in second repeat.

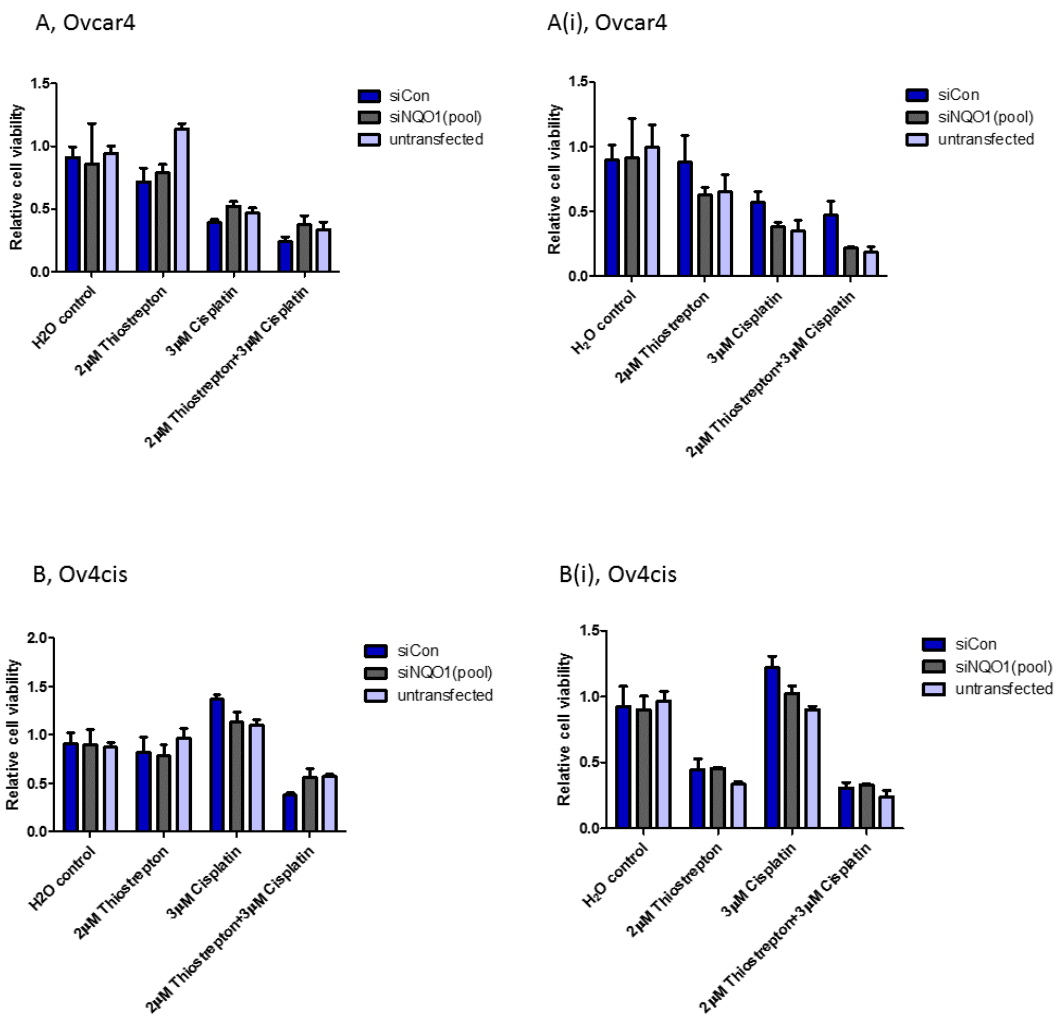
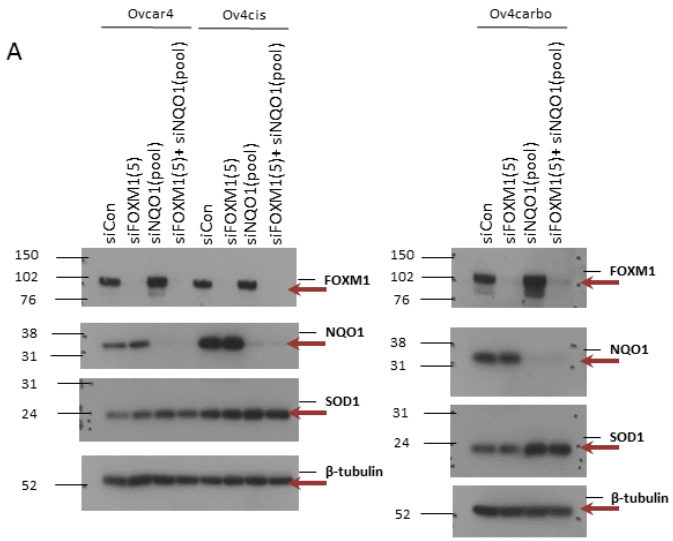


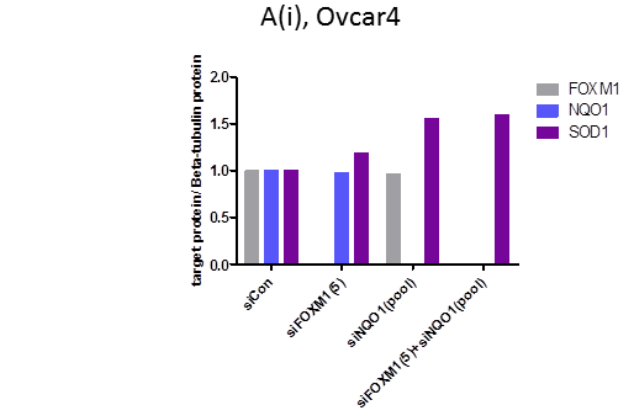
Figure 29. Thiostrepton sensitises ovarian cancer cells to Cisplatin.

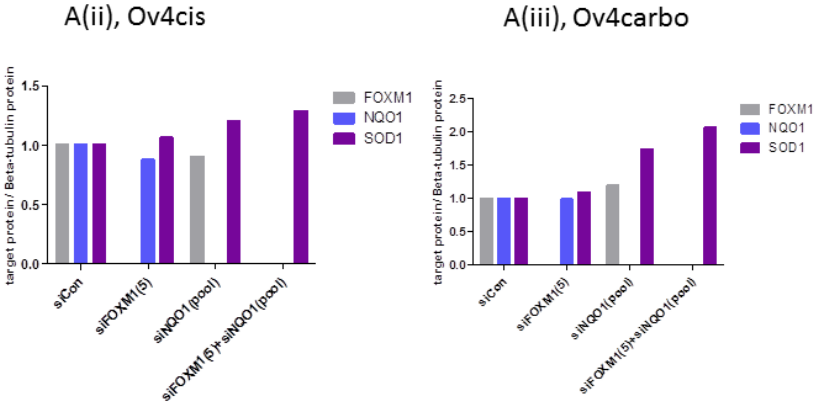
A) B) Cisplatin sensitisation was measured using CTG, post-treatment with 1μM Thiostrepton +/- 3μM Cisplatin, or vehicle control, after *Nqo1* silencing in a 96-well plate. Data was normalised to its vehicle control sample, H2O control. Two repeats are shown for each cell line, and error bars represent SD.

4.5 The effect of *Foxm1* + *Nqo1* silencing is not additional to *Nqo1* silencing, where the antioxidant enzymes SOD1 and Gpx1 have increased protein expression

Our previous data has suggested that SOD1 expression was also increased in the platinum-resistant ovarian cancer cells, therefore we analysed whether expression of SOD1 and a further enzyme in the NRF2-antioxidant response pathway, Gpx1, were influenced by changes in FOXM1 or NQO1 expression. We immunoblotted protein lysates that had been isolated from Ovcar4, Ov4cis and Ov4carbo ovarian cancer cell lines that had been transfected with either siRNA against FOXM1 or NQO1, alone or together, or a non-targeting siRNA control (siCon).


A
Ovcar4
Ov4cis
Ov4carbo
siCon
siFOXM1(5)
siNQO1(pool)
siFOXM1(5)+ siNQO1(pool)
150
102
76
38
31
24
52
FOXM1
NQO1
SOD1
β-tubulin


A(i), Ovcar4
target protein/ Beta-tubulin protein
2.0
1.5
1.0
0.5
0.0
FOXM1
NQO1
SOD1
siCon
siFOXM1(5)
siNQO1(pool)
siFOXM1(5)+siNQO1(pool)


A(ii), Ov4cis
A(iii), Ov4carbo
target protein/ Beta-tubulin protein
FOXM1
NQO1
SOD1
siCon
siFOXM1(5)
siNQO1(pool)
siFOXM1(5)+siNQO1(Pool)

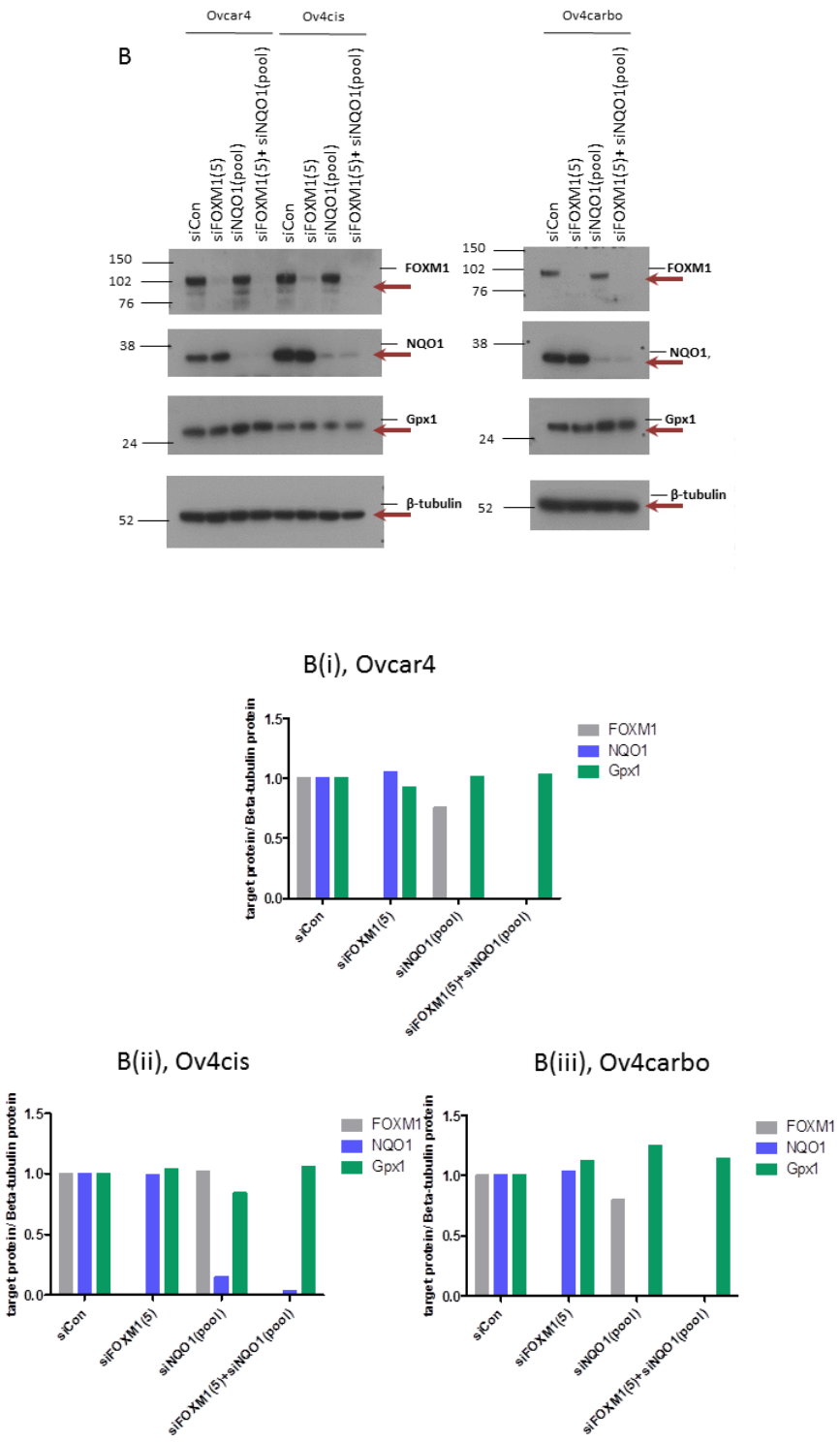


Figure 30. SOD1 and Gpx1 protein expression levels upon Foxm1 and Nqo1 double silencing in the ovarian cancer cell lines Ovcar4, Ov4cis and Ov4carbo.

A) Ai-iii) B) Bi-iii) Whole cell lysates collected 72-hours post-transfection with si*Foxm1*(5) and si*Nqo1*(pool), alone or together, were analysed on Western blot and probed with FOXM1, NQO1, SOD1 and Gpx1 antibodies. β-tubulin was used as

a loading control. The controls used for this transfection were siCon (negative control) and siPLK1 (positive control). N=1. Image J was used to quantify Western blots.

Interestingly, in the all cell lines, we observed an increase in the expression of SOD1, upon *Nqo1* silencing and this was maintained upon *Foxm1* + *Nqo1* silencing (Figure 30A). We also observed an increase in the expression of Gpx1, upon *Foxm1*/*Nqo1* silencing and upon *Foxm1* + *Nqo1* silencing, in Ov4carbo cells (Figure 30B), suggesting FOXM1 and NQO1 may be regulating the NRF2-antioxidant pathway in general.

5.0 Analysing the sensitivity of ovarian cancer cells to Thiostrepton and to Diminutol

5.1 Treatment with the FOXM1 inhibitor Thiostrepton and the NQO1 inhibitor Diminutol reduce cell viability

Based on our data demonstrating that silencing of *Foxm1* and *Nqo1* together reduced cell viability (Figure 26) and colony formation (Figure 27A-C) in our ovarian cancer cells, and the greatest in the Ovcar4 cells, we next wanted to understand the therapeutic implications of this and whether this could be exploited with clinical inhibitors of FOXM1 (Thiostrepton) and NQO1 (Diminutol). To this end, we first determined whether these drugs alone caused any effect on cell viability in our cells by treating cells with either increasing concentrations of the FOXM1 inhibitor Thiostrepton (Figure 31A) or the NQO1 inhibitor Diminutol (Figure 31B) for 72-hours, and analysed cell viability.

A range of Thiostrepton and Diminutol concentrations were tested to support further selection of concentrations in combination strategy experiments for Figures 32-34. Diminutol has a reported Ki of 1.72μM, as a competitive inhibitor of NQO1 [152]. Therefore, concentrations selected were also based on what is already known about the compound.

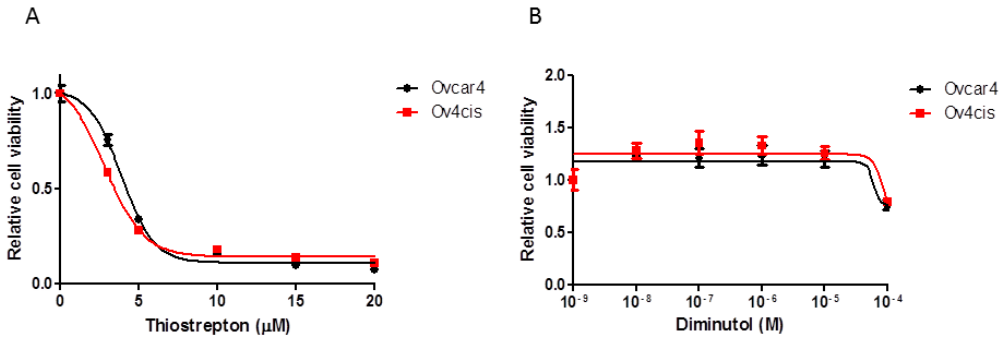


Figure 31. Sensitivity of ovarian cancer cells to Thiostrepton treatment and Diminutol treatment.

A) Dose response curves for Thiostrepton in ovarian cancer cells, after 72-hours treatment in a 96-well plate. Data was normalised to vehicle control. N=1 and error bars represent SD. B) Dose response curves for Diminutol in ovarian cancer cells, after 72-hours treatment in a 96-well plate. N=1 and error bars represent SD.

Table 8. IC50 values for ovarian cancer cells treated with Thiostrepton and Diminutol.

Cell line, compound	IC50 value (μM)
Ovcar4, Thiostrepton	3.9
Ov4cis, Thiostrepton	3.8
Ovcar4, Diminutol	Out of range
Ov4carbo, Diminutol	Out of range

Our results suggest that there was no difference in sensitivity to either Thiostrepton or Diminutol alone in the Ov4cis cells, in comparison to the parental Ovcar4 cells (Table 8).

6.0 A combined treatment of Thiostrepton and Diminutol show they more greatly reduce viability in ovarian cancer cells

6.1 Thiostrepton and Diminutol treatment in combination cause significantly reduced cell viability, compared to either agent alone

Previous investigations have shown that when Thiostrepton or Bortezomib were used in combination with ROS inducers, this caused apoptosis [187]. There is currently no published data on FOXM1 inhibitors being used in combination with NQO1 inhibitors. To investigate this, we treated Ovcar4 and Ov4cis cells with 2μM Thiostrepton, 10μM Diminutol, 2μM Thiostrepton + 10μM Diminutol or vehicle control and analysed cell viability after 96-hours. Our results suggested that treatment with Thiostrepton and Diminutol in combination caused a greater reduction in cell viability, compared to either agent alone (Figure 32A-B).

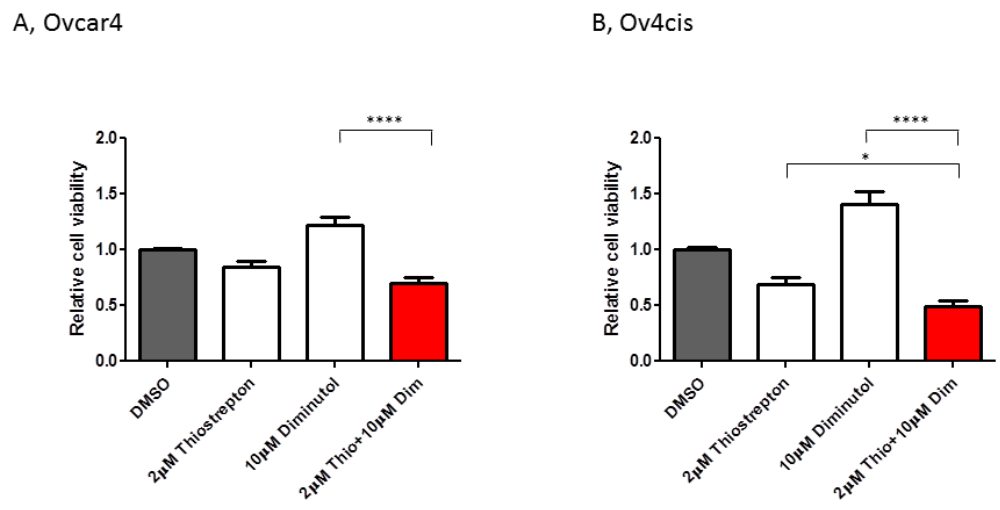


Figure 32. Treatment with Thiostrepton and Diminutol in combination cause a greater reduction in cell viability, compared to either agent alone, in ovarian cancer cells.

A) B) Cell viability was measured using CTG, at 96-hours post-treatment in a 96-well plate. The treatments used were 2μM Thiostrepton alone (white), 10μM Diminutol alone (white), 2μM Thiostrepton + 10μM Diminutol (red) and vehicle control, DMSO (grey). Data was normalised to its vehicle control sample, DMSO. N=3 and error bars represent SEM. T-tests were used (p-value of: <0.05*, <0.0001****).

Since both the Ovcar4 cells and the Cisplatin-resistant cells had reduced cell viability upon treatment with our combination therapy, this suggests that this treatment is independent of chemosensitivity as well as expression levels of FOXM1 and NQO1. Given the role of FOXM1 in regulating DNA repair and the antioxidant response pathway protein NRF2 [115, 302], reduced levels of this protein could be detrimental to cells by causing increased DNA damage and/or increased oxidative stress. Then, given the role of NQO1 as a ROS scavenger [141], reduced levels of this protein could also be detrimental to cells by causing increased oxidative stress. Taken together, this result suggests that targeting FOXM1 and NQO1 in cancer cells could have translational relevance, and that Thiostrepton and Diminutol could be an effective treatment option for cancer patients.

7.0 Investigating the mechanistic basis for Thiostrepton and Diminutol as a combination therapy

7.1 There are increased DNA double-strand breaks upon treatment with Thiostrepton and Diminutol, compared to either agent alone, in Cisplatin-resistant ovarian cancer cells

Having observed an increased reduction in cell viability upon treatment with Thiostrepton and Diminutol in combination (Figure 32A-B), we next investigated the mechanism for our treatment combination. The action of Thiostrepton is firstly on FOXM1, through inhibition, and subsequently on expression of various downstream proteins, including DNA repair protein expression [115]. So this led us to investigate the amount of DNA double-strand breaks in our cells, under our different treatment conditions. Previously, FOXM1-deficient cells have been shown to exhibit increased DNA breaks [115, 118]. We have also shown a significant increase in DNA double-strand breaks upon treatment with Thiostrepton and Olaparib, compared to either agent alone, in breast cancer cells (Results, Chapter 3).

We therefore determined whether Thiostrepton and Diminutol in combination caused enhanced DNA double-strand breaks in our cells, compared to either agent alone. To this end, we treated Ovcar4, Ov4cis and Ov4carbo cells with 2μM Thiostrepton, 5μM Diminutol, 2μM Thiostrepton + 5μM Diminutol or vehicle control. We analysed γH2AX foci, a marker of DNA double-strand breaks, after 48-hours and determined that DNA double-strand breaks were increased upon treatment with

Thiostrepton and Diminutol, compared to either agent alone, in the Ov4cis cells (Figure 33B).

In Ov4cis cells only, we observed an increase in DNA double-strand breaks in cells treated with Thiostrepton and Diminutol in combination, compared to either agent alone. Whereas, in Ov4carbo cells, there was a significant increase in DNA double-strand breaks upon treatment with Thiostrepton alone, but not in combination with Diminutol. This result could suggest that this mechanism of increase DNA double-strand breaks may be playing a lesser role in Ov4carbo cells, and needs to be further investigated. In Ovcar4 cells, the error bars are too large to make any conclusions about the response, and therefore needs to be repeated.

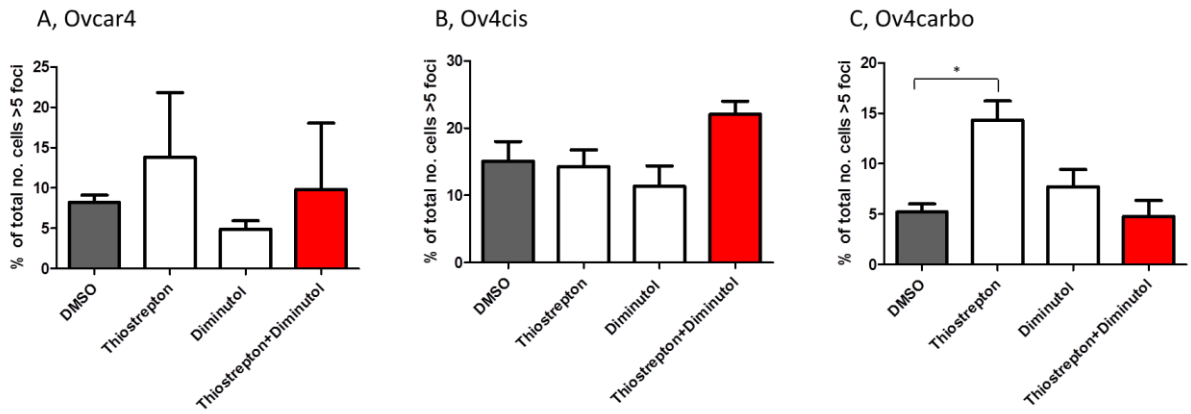


Figure 33. γH2AX foci in ovarian cancer cells upon treatment with Thiostrepton alone, Diminutol alone and Thiostrepton and Diminutol in combination.

A) B) C) Percentage of total number of cells containing more than 5 foci were measured using confocal microscopy, 48-hours post-treatment of cells on coverslips in a 24-well plate. The treatments used were 2µM Thiostrepton (white), 5µM Diminutol (white), 2µM Thiostrepton + 5µM Diminutol (red) and vehicle control, DMSO (grey). N=2 and error bars represent SEM. T-tests were used (p-value of: <0.05*).

Our results suggest that increased DNA double-strand breaks may be contributing to the mechanism of action of our combination therapy. This does not exclude the possibility that other mechanisms may also be acting in cells treated with Thiostrepton and Diminutol in combination.

7.2 There is a significant increase in reactive oxygen species upon treatment with Thiostrepton and Diminutol, compared to either agent alone, in Cisplatin-resistant ovarian cancer cells

To further investigate the mechanism for our treatment combination, we next considered reactive oxygen species production in our cells, under our different treatment conditions. Previously, melanoma cells were examined for upregulation of cellular oxidative stress in response to 10μM Thiostrepton, for 1- to 6-hour exposure, where there was around five-fold increase in ROS within an hour of exposure to Thiostrepton [326]. FOXM1-deficiency has also been shown to cause elevated ROS levels [119]. Whilst lung adenocarcinoma cells examined after NQO1 depletion showed increased endogenous ROS [327], and pancreatic cancer cells treated with the NQO1 inhibitor Dicoumarol induced growth inhibition through increased ROS production [328]. Conversely, prostate cancer cells examined after inhibition of NQO1 showed decreased endogenous ROS [329], and cholangiocarcinoma cells treated with the NQO1 inhibitor Dicoumarol showed no increase in ROS formation [158]. These differences in ROS generation could be cell-specific, and NQO1 inhibition could exert different cell killing mechanisms. Furthermore, regulation through the ROS response may be selective.

Therefore, given the extensive literature linking FOXM1 and NQO1 inhibition to ROS generation, we determined whether Thiostrepton and Diminutol in combination caused enhanced reactive oxygen species production in our cells, compared to either agent alone. To this end, we treated Ovcar4 and Ov4cis cells with 10μM Thiostrepton, 10μM Diminutol, 10μM Thiostrepton + 10μM Diminutol or vehicle control. We analysed production of reactive oxygen species, a marker of oxidative stress, after 3-hours and determined that there was a trend for increased ROS generation upon treatment with Thiostrepton and Diminutol, compared to either agent alone, in the Ov4cis cells (Figure 34B). Our results therefore suggest our combination therapy may also affect ROS generation.

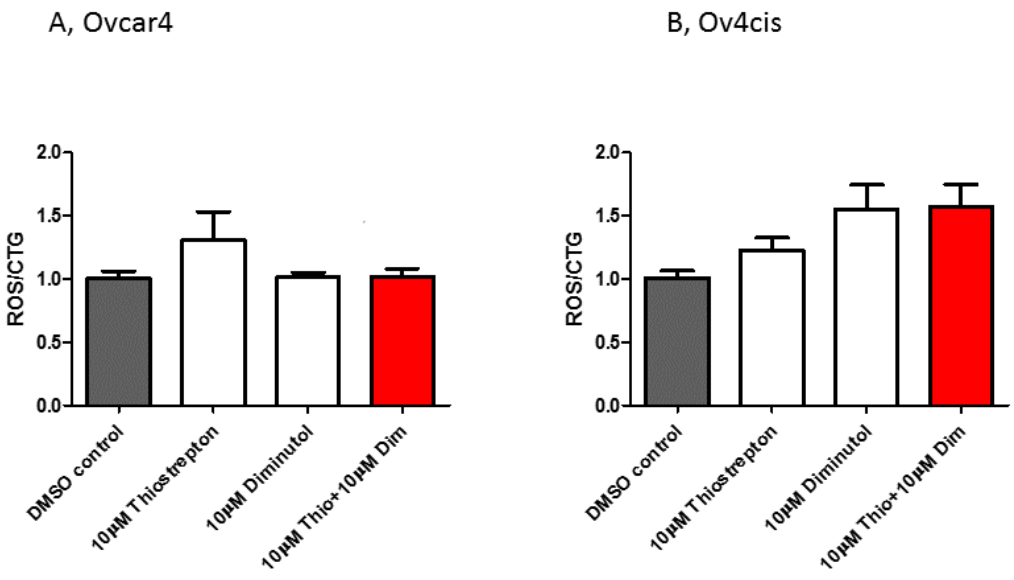


Figure 34. ROS production in the ovarian cancer cells upon treatment with Thiostrepton alone, Diminutol alone and Thiostrepton and Diminutol in combination.

A) B) ROS levels (fluorescence), normalised to cell viability (luminescence), were measured 3-hours post-treatment in a 96-well plate. The treatments used were 10μM Thiostrepton (white), 10μM Diminutol (white), 10μM Thiostrepton + 10μM Diminutol (red) and vehicle control, DMSO (grey). Data was normalised to its vehicle control sample, DMSO. N=1 and error bars represent SD.

8.0 Investigating the therapeutic implications of combining Thiostrepton and ROS-inducing agents

8.1 A combined treatment of Thiostrepton and ROS-inducing agents show they cause a more greatly reduced cell viability in breast cancer cells

Alongside our investigation of FOXM1, NQO1 and chemoresistance, we also investigated the therapeutic implications of combining the FOXM1 inhibitor Thiostrepton and ROS-inducing agents in breast cancer cells. Using this approach, we were able to explore other combination treatment options. We could also confirm previously published data for Thiostrepton or Bortezomib used in combination with ROS inducers to cause apoptosis [187].

We began by treating cells with the ROS-inducing agents, Menadione and Podophyllotoxin in dose responses, and determined if our cells responded differently to each ROS-inducing agent. To this end, we treated our cells with increasing concentrations of Menadione (Figure 35A) and Podophyllotoxin (Figure 35B). A range of Menadione and Podophyllotoxin concentrations were tested to support further selection of concentrations in combination strategy experiments for Figures 36-37. Menadione has been shown to induce ROS in breast cancer cells at 5μM [330]. Furthermore, a Podophyllotoxin derivative has been shown to induce ROS in breast cancer cells at 10μM [331]. The literature also guided our decision about which concentrations to test. This was considered alongside compound toxicity.

We analysed cell viability after 72-hours, and determined that HCC1954 and MCF7 were more sensitive to the ROS-inducing agent Podophyllotoxin than Menadione (Table 9).

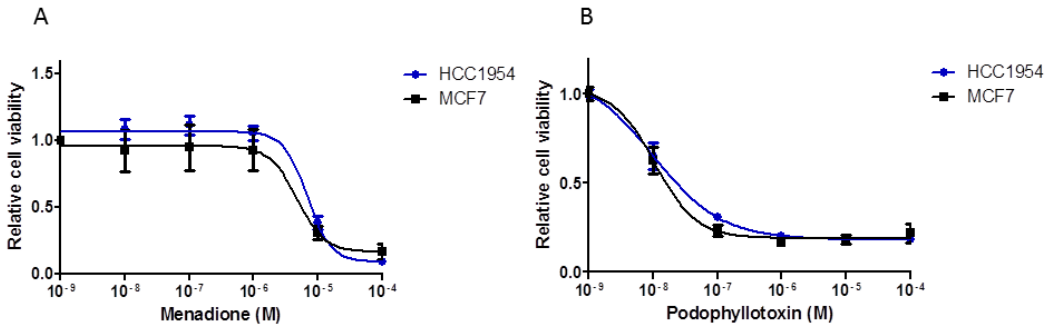


Figure 35. Sensitivity of breast cancer cells to the ROS-inducing agents, Menadione and Podophyllotoxin.

A) Dose response curves for Menadione in breast cancer cells, after 72-hours treatment in 96-well plate. Data was normalised to its vehicle control. N=2 and error bars represent SE. B) Dose response curves for Podophyllotoxin in breast cancer cells, after 72-hours treatment in 96-well plate. N=3 and error bars represent SE.

Table 9. IC50 values for breast cancer cells treated with Menadione and Podophyllotoxin.

Cell line, compound	IC50 value (μM)
HCC1954, Menadione	7.0
MCF7, Menadione	4.8
HCC1954, Podophyllotoxin	0.009
MCF7, Podophyllotoxin	0.01

We then tested ROS-inducing agents in combination with Thiostrepton to explore other potential combination therapy options for patients. In the first instance, we used the FOXM1 inhibitor Thiostrepton and the ROS-inducing agent Menadione. To this end, we treated HCC1954 with 50nM Thiostrepton, MCF7 with 500nM

Thiostrepton, both cell lines with 8μM or 5μM Menadione, combination options or vehicle control, on day 2 and day 7. We analysed cell viability on day 10, and determined that Thiostrepton and Menadione in combination caused a greater reduction in cell viability, compared to either agent alone (Figure 36A-B).

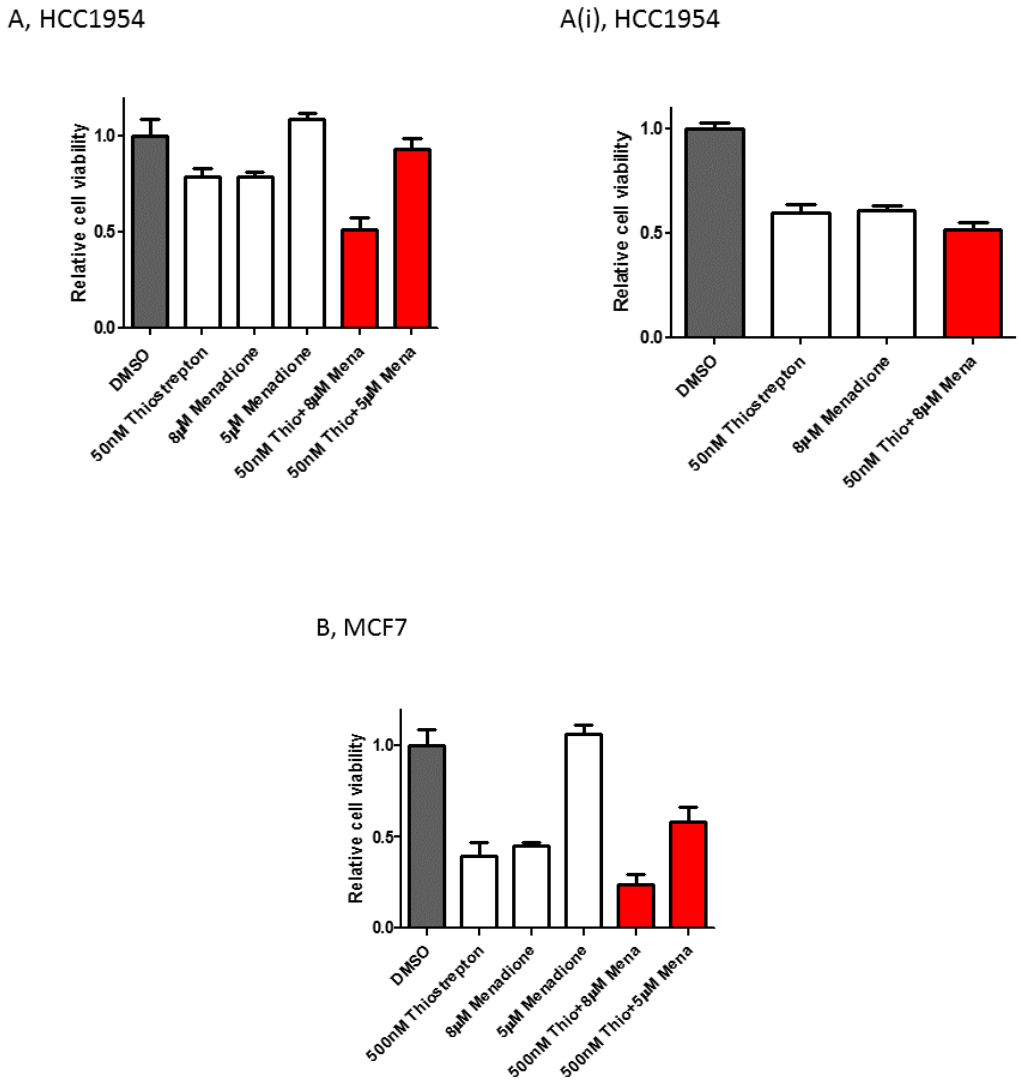


Figure 36. Treatment with Thiostrepton and Menadione in combination cause greater reduction in cell viability, compared to either agent alone, in breast cancer cells.

A) Ai) B) Cell viability was measured using CTG, on day 10 post-treatment in a 96-well plate. The treatments used were Thiostrepton alone (white), Menadione alone (white), Thiostrepton and Menadione in combination (red), and DMSO-vehicle control (grey). For HCC1954, the treatments were 50nM Thiostrepton alone, 8μM

Menadione alone, 5µM Menadione alone, 50nM Thiostrepton + 8µM Menadione, 50nM Thiostrepton + 5µM Menadione and vehicle control. The treatments used for MCF7 were 500nM Thiostrepton alone, 8µM Menadione alone, 5µM Menadione alone, 500nM Thiostrepton + 8µM Menadione, 500nM Thiostrepton + 5µM Menadione and vehicle control. Data was normalised to its vehicle control sample, DMSO. Two repeats shown (HCC1954) and N=1 (MCF7) is shown and error bars represent SD.

The FOXM1 inhibitor Thiostrepton was also combined with the ROS-inducing agent Podophyllotoxin. To this end, we treated HCC1954 with 40nM Thiostrepton, MCF7 with 50nM Thiostrepton, both cell lines with 10nM Podophyllotoxin, combination options or vehicle control, on day 2, day 5 and day 8. We analysed colony formation on day 11 and determined that Thiostrepton and Podophyllotoxin in combination caused a more significantly reduced colony formation, compared to either agent alone (Figure 37A-B).

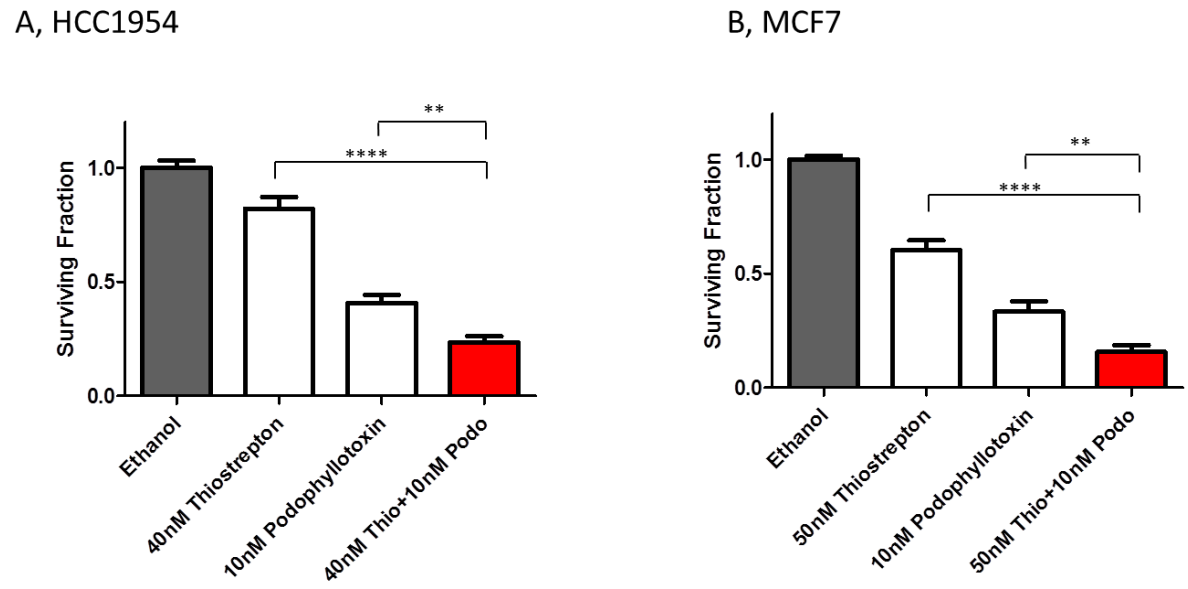


Figure 37. Treatment with Thiostrepton and Podophyllotoxin in combination cause a greater reduction in colony formation, compared to either agent alone, in breast cancer cells.

A) B) Colony formation was measured using SRB staining and counting colonies, on day 11 post-treatment in a 6-well plate. The treatments used were Thiostrepton alone (white), Podophyllotoxin alone (white), Thiostrepton and Podophyllotoxin in combination (red), and ethanol-vehicle control (grey). For HCC1954, the treatments

were 50nM Thiostrepton alone, 10nM Podophyllotoxin alone, 50nM Thiostrepton + 10nM Podophyllotoxin and vehicle control. The treatments used for MCF7 were 40nM Thiostrepton alone, 10nM Podophyllotoxin alone, 40nM Thiostrepton + 10nM Podophyllotoxin and vehicle control. Data was normalised to its vehicle control sample, ethanol. N=3 and error bars represent SEM. T-tests were used (p-value of: <0.01**, <0.0001****).

Our data suggests that Thiostrepton and the ROS-inducing agents, Menadione and Podophyllotoxin, can be used in combination in a therapeutic context. These combinations could be used as an alternative treatment option for cancer patients.

Taken together, we have confirmed that FOXM1 plays a role in the oxidative stress response, and that this role can be exploited therapeutically. Our data suggests that there may be a regulatory relationship between FOXM1 and NQO1, and upon depletion of these proteins together, we observed a greater reduction in cell viability and colony formation in ovarian cancer cells. Furthermore, we have suggested that FOXM1 and NQO1 may be involved in driving Cisplatin resistance in ovarian cancer cells. Therapeutically, we have shown that the FOXM1 inhibitor Thiostrepton and the NQO1 inhibitor Diminutol could be offered as a potential combination therapy to help treat cancer patients.

Discussion

Chapter 5

1.0 Thiostrepton and Olaparib are synthetically lethal in breast cancer

1.1 FOXM1 in normal cells and cancer cells

FOXM1 plays an extensive number of roles in the cell. These roles are essential to many fundamental cellular pathways, including cell cycle regulation, DNA damage response regulation and oxidative stress response regulation, where FOXM1 is itself regulated through auto-regulation and post-translational modifications [98, 105, 115, 199]. Therefore, FOXM1 has functional significance in normal cells, and it is these activities have been harnessed by cancer cells by increasing expression of this transcription factor [180].

Overexpression of FOXM1 has been identified in several solid tumours, and it has been subsequently recognised as a tumour biomarker [180]. Poor prognosis has also been linked to patients with higher expression of FOXM1 [169]. In studies of specific cancer types, such as breast cancer, HER2 status was found to be correlated with FOXM1, where FOXM1 could be used as a potential target in resistant breast cancer tumours [168]. Not only have cancer cells been shown to have higher FOXM1 expression, this increased FOXM1 expression has also been implicated in chemoresistance [191].

Taken together, FOXM1 represents a novel candidate in cancer therapy, and its role in chemoresistance emphasises a greater need for developing novel treatment strategies for cancer patients. It is also important that a patient's normal cells may be spared in this treatment approach, so that only the cancer cells are susceptible to the cancer therapy.

1.2 FOXM1 and homologous recombination

This project first proposed to exploit the role of FOXM1 in homologous recombination, using a combination therapy to achieve a synthetic lethal approach, with translational application [116-118]. The success demonstrated by existing clinically relevant synthetic lethal approaches, such as those of PARP inhibitors in *Brca1*/*2*-deficient cancers, as well as several other studies that have identified synthetic lethal combinations, such as MMR-deficiency and Methotrexate, led us to apply a similar approach in the context of targeting FOXM1 and reduced homologous recombination [40, 299].

To expand on the topic of synthetic lethal approaches, in view of other successes and possible limitations, another example includes the identification of 3′-phosphoadenosine 5′-phosphosulfate (PAPS) synthase 1 (PAPSS1) as a novel platinum-sensitising target in non-small cell lung cancer [332]. The clinical potential of this study is that it addresses an existing treatment problem in advanced non-small cell lung cancer patients, where the standard platinum-based chemotherapy response rate is only 30–33% [332]. A limitation of this current study, however, is that an siRNA approach to targeting PAPSS1 was used, and this would be difficult to deliver in therapeutically relevant doses [332]. Therefore, validating potent PAPSS1 inhibitors for use as a therapeutic agent would be required as the next step [332]. Overall, this study is also relevant to our project, such that it shows how we can use siRNA and compounds as two experimental approaches, to demonstrate and validate synthetic lethality in cancer cells.

Since FOXM1 has been shown to directly regulate HR genes, including *Rad51*, *Brip1* and *Nbs1*, we were first of all interested to determine whether we could

correlate FOXM1 with HR proteins in different panels of cancer cell lines [116-118]. Since RAD51 is a central enzymatic component of HR, we decided to correlate expression levels of FOXM1 with expression levels of RAD51. In one study, the role of RAD51 in tumourigenesis was investigated [333]. The authors found that cells expressing a dominant-negative form of RAD51 generated more tumours, with faster growth, compared to control and wild-type mammalian RAD51 cells [333]. That said, overexpression of RAD51 stimulated HR, and induced strong chromosome instability, and this showed that there are risks of excess HR [334]. Altogether, this data has indicated the importance of tight control of the level of HR.

As our later mechanistic data also showed, where *Foxm1* silencing reduced expression levels of HR genes, alongside this initial Western blot correlation, we were able to confirm current findings that have been previously discussed, which have demonstrated FOXM1 as a transcriptional regulator of genes involved in HR. Then, going further, our data showed for the first time that *Rad51* is regulated by FOXM1 in breast cancer cells, having only previously been shown in glioblastoma cells [116]. Our data also revealed the potential extent at which FOXM1 regulates members of the HR pathway in breast cancer cells, where *Rad51* and *Brip1* mRNA levels seemed more reduced upon *Foxm1* silencing, compared to our other investigated genes, *Brca2* and *Nbs1*. This data will be further considered later on in our discussion, once our combination therapy has been discussed.

To investigate the relationship between FOXM1 and RAD51 in a more clinically relevant setting, we used patient data and observed that higher FOXM1 expression and higher RAD51 expression both, individually, correlated with a reduced survival probability. Again, our data validated the earlier findings in glioblastoma patients

[116], this time in breast cancer patients. It may be of added benefit to explore the relationship between FOXM1 and RAD51 together to add to what we have investigated here, which was individually, in patients with reduced survival probability. Furthermore, it may also be of benefit to investigate the correlation of FOXM1 and RAD51 in breast cancer samples using immunohistochemical analyses, as carried out in recurrent glioblastoma samples [116]. Overall, we could use additional patient data to supplement the *in vitro* data that we have obtained.

1.3 Selecting compounds for our combination therapy

After our initial investigations showed a relationship between FOXM1 and RAD51 in breast cancer cells that could be further explored, this prompted us to next test a combination treatment strategy in these cells. This would be on the basis of exploiting targeting FOXM1 and HR, in cancer cells, whilst sparing a patient's normal cells. Our combination strategy was first on our experimental priorities so that we could develop a therapy that would have translational benefit to cancer patients. Once established, we then intended to go on and investigate the possible mechanistic implications of our combination therapy.

This combination therapy was in fact very much a case of applying what has been successfully, and clinically, achieved with PARP inhibitors. Instead of exploiting a germline *Brca1/2* mutation or "BRCAness" in tumours, which causes ineffective HR and therefore leads to increased sensitivity to PARP inhibitors and ultimately selective cell death, our approach was almost to mimic this effect upstream of this pathway, by targeting an upstream regulator of HR genes [277]. So, by targeting

FOXM1, this would in turn reduce expression levels of its downstream targets, including HR genes, and then FOXM1-targeted cancer cells could be sensitised to PARP inhibition, and induce selective cell death.

As alluded to previously, the concept of targeting FOXM1 as part of a combination therapy is not entirely new. In fact, at the time of our study, there were other groups working on similar concepts to us. In particular, the authors of one of the most recent of these studies were able to show that targeting FOXM1 with Thiostrepton induced “BRCAness” and enhanced sensitivity to Olaparib, in ovarian cancer cells [310]. We have shown for the first time that this synthetic lethality approach can occur in breast cancer cells. It is also important to note that this work provided some insight into the adaptive response of ovarian cancer cells to PARP inhibition, relating to the emergence of chemotherapy resistance that is often observed in ovarian cancer patients [310]. The adaptive cellular response has been suggested to provide a transitional state for cells to acquire fitness-conferring genetic mutations, after several rounds of treatment with chemotherapeutic agents, and the authors have implicated FOXM1-mediated adaptive pathways to be involved in this response [310]. Furthermore, regarding the mechanism of action of PARP inhibitors, as previously discussed, the authors observed that Thiostrepton caused an enhanced amount of DNA damage and PARP1 trapping in cells treated with Olaparib [310]. This was referred to as being a further mechanistic explanation for increased sensitivity to Olaparib, as PARP trapping has been considered to be more cytotoxic than inhibition of the enzymatic activity of PARP [289, 310]. Upon PARP trapping, PARP1 is trapped on DNA by the PARP inhibitor, and PARP1-DNA complexes could then interfere with DNA replication, and this mechanism therefore explains why despite similar potencies to inhibit PARP catalytic activity, PARP trapping can affect the toxicity of different PARP inhibitors [289].

Other studies demonstrated that targeting FOXM1 induced Cisplatin sensitivity in ovarian cancer cells [308]. This study is clinically relevant to ovarian cancer patients because chemotherapy represents a standard of care treatment option for these patients, and emergence of chemoresistance to Cisplatin is a problem for successful treatment [308]. FOXM1 was shown to be higher in patient samples that were Cisplatin-resistant, compared to Cisplatin-sensitive, and when FOXM1 was targeted this increased sensitivity to Cisplatin [308]. As EXO1 was identified to be regulated by FOXM1 and DNA repair was promoted upon Cisplatin treatment, it was suggested that targeting FOXM1 and EXO1 could improve Cisplatin sensitivity in patients [308]. This study provides a potential parallel with our data. While EXO1 is the only DNA repair protein implicated in this study of Cisplatin sensitivity, other DNA repair proteins, such as those included in our project, may also contribute to Cisplatin resistance. Therefore, FOXM1 inhibition in combination with PARP inhibition may provide a potential therapeutic opportunity to overcome Cisplatin resistance in cancer cells.

The choice of FOXM1 inhibitors and PARP inhibitors are extensively covered in the Introduction, and indeed many of those listed represent suitable candidates to be used in our combination treatment strategy. As our study had a principle focus on identifying a novel treatment combination with translational benefit in breast cancer patients, it was certainly of consideration to select two compounds that are already FDA-approved so that they could therefore enter clinical trials at a more advanced stage of the drug discovery process.

Of the FOXM1 inhibitors previously discussed, a number of studies that have been conducted using these compounds to inhibit FOXM1, yet there are differing insights

into the mechanistic activity of these compounds. Then, it is also worth considering that the thiazole antibiotics Siomycin A and Thiostrepton were the first chemical inhibitors of FOXM1 to be identified [183, 194, 198]. While proteasome inhibitors, such as Bortezomib, and small molecule inhibitors were next identified [195].

When the mechanism of action of these FOXM1 inhibitors was explored, Thiostrepton has been subjected to a more in-depth understanding, compared to the other compounds. In an early study, Siomycin A was suggested to act by at least two mechanisms when antagonising FOXM1 [194]. This included blocking its phosphorylation, thereby causing reduced transactivation ability, and down-regulation of FOXM1 mRNA and protein levels [194]. However, this was later subjected to a certain level of scrutiny in a correspondence which indicated that a complete model for the mechanism of action of thiazole antibiotics was still lacking, at that time [335]. In particular, the correspondence mentioned how the effect of Siomycin A treatment on non-transformed cells had not yet been investigated, and that this may have important implications for the mechanism of action of Siomycin A [335].

Studies have shown that Thiostrepton specifically inhibited proliferation of MCF7 breast cancer cells, but not MCF10A breast epithelial cells, suggesting that Thiostrepton does not promote a general cytotoxic phenotype [183]. It also showed that Thiostrepton inhibited FOXM1 protein and mRNA expression, and that FOXM1 expression was inhibited predominantly at the gene promoter level [183]. Therefore, these authors have suggested that FOXM1 may be a target of Thiostrepton because of decreased FOXM1 mRNA levels upon Thiostrepton treatment, which may also be supported by the proposed auto-regulatory mechanism of FOXM1 itself

[183, 199]. In a later study, Thiostrepton was shown to directly bind to the FOXM1 protein, thus preventing its interaction with several gene promoters, but not necessarily to its own [203].

In another study, Thiostrepton's suggested activity of inhibiting transcriptional activity and FOXM1 expression via proteasome inhibition was considered [204]. As proteasome inhibitors act to stabilise cellular proteins, this seemingly counterintuitive model was further investigated [205]. Therefore, in this model, proteasome inhibitors were suggested to increase expression of a putative negative regulator of FOXM1, and this putative negative regulator was later identified as HSP70 [204, 205].

The proteasome inhibitor Bortezomib has also been identified as a FOXM1 inhibitor, and it has been shown to inhibit FOXM1 transcriptional activity and FOXM1 expression [195]. The selectivity of Bortezomib has not yet been discussed in the context of studies that have investigated its role as a FOXM1 inhibitor. That said, Bortezomib is a 26S proteasome inhibitor that is used in the treatment of relapsed/refractory, relapsed, and newly diagnosed multiple myeloma (MM), and a study has demonstrated that Bortezomib significantly down-regulated IκBα expression and triggered NF-κB activation in MM cell lines and primary tumour cells from MM patients [336]. Furthermore, as mentioned in the introduction, Bortezomib was the first proteasome inhibitor to enter clinical practice [206]. Bortezomib has also been used in a study as a potential inhibitor of oestrogen receptor-positive breast cancer, where FOXM1 was among a list of genes down-regulated by this compound [337]. It was also suggested that FOXM1 may mediate the effects of Bortezomib on multiple genes [337]. Despite the potential benefits of using this

compound in our studies, it may be more challenging to introduce this clinically because of the previously discussed restrictions on Bortezomib, such as dose limiting toxicity [206].

Collectively, based on the studies discussed, Thiostrepton has received more coverage in the current literature, compared to other FOXM1 inhibitors. This can be further emphasised by the extensive use of Thiostrepton in studies of different cancer types including, human cancer cells testing different lines [198], nasopharyngeal carcinoma cells [338], medulloblastoma cells [201], and ovarian cancer cells [310]. So, together with the mechanistic studies of Thiostrepton that showed selectivity for MCF7 breast cancer cells, compared to MCF10A breast epithelial cells, as well as the model for direct interaction of Thiostrepton with FOXM1, and the use of Thiostrepton in different cancer types, made this the compound of choice for our study.

Whilst the studies previously discussed highlight the several benefits to using Thiostrepton as a FOXM1 inhibitor, there still exist potential limitations when translating this to human treatment. This was referred to by Fang and colleagues, where they mentioned that whilst it has current use as a topical antibiotic, human use could be limited by the low solubility and bioavailability [310]. A study has shown that micelle-formulated Thiostrepton had better solubility and pharmacodynamic effect on the tumour in xenograft models [310, 339]. Therefore, Thiostrepton may still represent a highly suited compound for treatment of human cancer, when formulated into nanoparticles [339].

Of the PARP inhibitors discussed, the selection was more straightforward than that of the FOXM1 inhibitors. This was more to do with the fact that PARP inhibitors currently offer huge clinical benefits to cancer patients. Several PARP inhibitors have been identified, and Olaparib represents the first PARP inhibitor to become available in clinical practice [40]. It has consequently been used in a number of studies and clinical trials, and its use has not been restricted to ovarian cancer patients, as it has also been shown to benefit breast cancer patients [39]. Olaparib has been used to treat patients with platinum-sensitive, relapsed, high-grade serous ovarian cancer, and has shown to significantly improve progression-free survival when used as a maintenance treatment [340]. Among patients with HER2-negative metastatic breast cancer and a germline *Brca* mutation, Olaparib monotherapy was demonstrated to have a significant benefit over standard therapy [39]. In another study, Olaparib as a monotherapy was evaluated in patients with *Brca1*/*2*-associated cancers, and encouraging data was shown across different cancer types which could be followed up in phase III trials [341]. Therefore, PARP inhibitors as a treatment option for patients may not only be limited to breast cancers and ovarian cancers associated with germline *Brca1*/*2* mutations, but may also be offered to other cancer patients [341].

In the FDA approval summary of Olaparib, treatment with Olaparib is indicated to give a better response rate and a favourable safety profile, compared to single agent chemotherapies that are available [40]. It is also stated that this compound has a relatively mild safety profile, rather than facing the cumulative toxicity from other treatment options [40]. Additionally, Olaparib is orally available, providing ease of administration, and has rapid absorption [40]. Clinical management of patients receiving Olaparib has also been investigated, and this is important to understand so that more supportive care can be offered to patients, caregivers, and health care

providers [342]. The provision of such documentation details some of the adverse effects associated with Olaparib treatment, and how dose modification may be an important consideration during therapy [342]. For example, fatigue may be experienced because of baseline disease-related fatigue, and gastrointestinal symptoms from disease burden, so awareness of these adverse effects can be effectively managed to allow therapy to continue [342]. Therefore, as a result of the extensive investigations of Olaparib, this compound was selected to be used in combination with Thiostrepton in our study.

We were able to show for the first time that Thiostrepton and Olaparib caused greater reduction in cell viability and colony formation, compared to either agent alone, in breast cancer cells, using respective assays. Our results indicated that FOXM1 inhibition using Thiostrepton enhanced sensitivity to the PARP inhibitor Olaparib, as shown by two different experimental approaches.

In terms of the benefits of our combination therapy compared to current standard of care treatments, the main aim of our research was to develop a novel combination therapy that can be offered as a treatment option to breast cancer patients. In the case of the study where Thiostrepton and Olaparib as a combination treatment strategy in ovarian cancer cells had been investigated, this was to overcome the adaptive cellular response and acquired resistance to chemotherapy [310]. Therefore, further investigation would be required to determine whether our combination therapy in breast cancer cells would offer the same outcome for patients who have chemoresistance. Nevertheless, our combination therapy was above all developed for the purpose of having translational benefit to breast cancer patients.

To be pursued clinically, the first major considerations would need to be reflective of current clinical data collected on patients who have already received treatment with Olaparib, and then how Thiostrepton may be used in a combination setting with this PARP inhibitor. Other major considerations would need to be inclusive of time- and dose-specific factors, to be tailored to individual patients.

1.4 Mechanistic implications of Thiostrepton and Olaparib as a treatment, and sparing normal cells

Once our combination therapy had been established we then decided to explore the mechanism of selectivity in cancer cells, compared to normal cells. By conducting *Foxm1* silencing assays and time course assays, this enabled us to gain a more detailed understanding of how FOXM1 inhibition was mediating sensitisation to Olaparib.

As previously mentioned, *Foxm1* silencing reduced expression levels of HR genes, as measured by RT-qPCR, and this allowed us to determine the extent at which HR gene expression was affected in FOXM1-depleted breast cancer cells. This data may in turn provide insight into how Thiostrepton mediates sensitisation to Olaparib, so that the therapeutic implications of this treatment combination can be evaluated for clinical benefit to cancer patients.

Whilst our *Foxm1* silencing data showed significantly decreased mRNA expression of all HR genes tested, we observed that *Rad51* and *Brip1* were reduced more consistently than *Brca2* and *Nbs1*, across our breast cancer cell lines. This result therefore suggested that RAD51 and BRIP1 could be under greater influence of

FOXM1. Since siRNA-mediated silencing is through mRNA degradation, while the mechanism of action of Thiostrepton is suggested to be through binding FOXM1 and preventing the regulation of other gene promoters [203], we used this data to guide our mechanistic understanding of how breast cancer cells may become sensitised to Olaparib, after Thiostrepton treatment.

In accordance with the previously discussed importance of testing Thiostrepton's selectivity in breast cancer cells, compared to normal breast epithelial cells, we too assessed the selectivity of the response in our breast cancer cell lines, and our normal breast epithelial cell line [183].

Firstly, regarding expression levels of FOXM1, we observed that FOXM1 expression levels, mRNA and protein, decreased at an earlier time-point of Thiostrepton treatment in HCC1954 breast cancer cells, compared to MCF10A normal breast epithelial cells. This observation was somewhat different to what had been previously shown by Kwok and colleagues, where they suggested that Thiostrepton had no effect on FOXM1 expression in untransformed MCF10A cells [183]. Interestingly, this group had treated MCF10A cells with 10μmol/L Thiostrepton for up to and including, 72-hours [183]. So whilst they did not treat for the same duration of time as our assay, 120-hours, they had also used a considerably higher concentration of Thiostrepton, as we used 50nM Thiostrepton. Overall, this differing response to Thiostrepton treatment observed between our work and that of Kwok and colleagues could be attributed to different basal levels of FOXM1 to begin with in the MCF10A cell lines [183]. This could in turn be an important consideration for patients receiving our combination therapy, to spare their normal cells.

Since our HCC1954 breast cancer cells responded earlier to Thiostrepton treatment, through decreased FOXM1 expression levels, compared to our MCF10A normal breast epithelial cells, this could open up a therapeutic window in a clinical setting. This early response of HCC1954 breast cancer cells suggests that treatment would need to be tailored to the patient, in a time-specific manner, so that Thiostrepton would only cause FOXM1 inhibition in cancer cells, thus sparing normal cells.

Referring back to the work of Kwok and colleagues, they observed a decrease in FOXM1 expression over the course of their time-points in MCF7 breast cancer cells, whilst we observed an initial decrease in FOXM1 expression in HCC1954 breast cancer cells, which was subsequently rescued over time [183]. These differences have therefore further illustrated the greater need for time- and dose-specific considerations, in a clinical setting. Then, as this is a combination therapy, the additional benefits of this strategy would be to use lower doses to reduce compound toxicity in patients.

In further view of the mechanistic implications relating to the involvement of HR genes in mediating Olaparib sensitisation after Thiostrepton treatment, the expression changes observed over time with Thiostrepton treatment were not completely aligned with our *Foxm1* silencing data. Notably, *Brip1* expression remained higher than other HR genes at the time-point where *Foxm1* expression was at its lowest. Yet, *Rad51* expression was reduced to a similar level as the other HR genes tested, *Brca2* and *Nbs1*.

Indeed, RAD51 could therefore play a more central role in mediating Olaparib sensitisation, after Thiostrepton treatment. This does not, however, exclude the possibility that other HR genes may also contribute to this sensitisation, albeit to a potentially lesser extent than RAD51. The implications of this could be viewed in different ways. In one instance, as RAD51 is fundamental to orchestrating the HR pathway, reducing expression levels of this particular protein with Thiostrepton treatment could assume a potentially greater sensitisation to Olaparib. This comparing to other less important proteins involved in the HR pathway, such that if their expression levels were reduced by treatment with Thiostrepton, the sensitisation to Olaparib may be lesser. In another instance, the repair mechanism choice during HR may also be considered, as this is critical in ensuring repair is as efficient and risk-free as possible [343]. In a study conducted in yeast cells, the authors described a model explaining how different repair mechanisms are able to compensate for each other during DSB repair [343]. They also referred to a balance between potentially competing DSB repair pathways [343]. Based on this study, it could be conceivable to suggest that cancer cells may exploit this kind of compensation and balance between repair mechanisms to their advantage, both in normal survival, and in overcoming treatment pressures such as our proposed combination therapy. Interestingly, another study has shown that upregulation of the HR pathway is capable of rescuing the growth and DNA repair defects associated with loss of BRCA1 function [344]. In particular, RAD51 was found to be upregulated, and shown to be a feature of BRCA1-deficient breast tumours [344]. This study therefore demonstrates that the expression levels of other proteins involved in HR can be altered when one protein becomes deficient in cells. Overall, these studies show how there may be changes in pathways and expression levels of other proteins to compensate for other changes in cells, such as the observed reduction in expression levels of HR proteins upon treatment with our combination therapy, Thiostrepton and Olaparib. Therefore, the implications here suggest that

cancer cells may have an enhanced sensitisation to Olaparib through reduced expression levels of RAD51, compared to other HR proteins. That said, cancer cells may adapt over time to our combination therapy, by employing compensatory mechanisms, and this could ultimately lead to the emergence of treatment resistance.

1.5 Significance of increased DNA damage in combination therapy

1.5.1 Decreased RAD51 may be mediating sensitisation to Olaparib

Having evaluated which HR genes may be involved in mediating sensitisation to Olaparib, after Thiostrepton treatment, our studies of DNA damage, specifically DNA breaks, enabled us to gain even more insight of the mechanistic action of the compounds together, compared to either agent alone. As mentioned in the introduction, FOXM1's role in the DNA damage response was not only illustrated by demonstrating that it transcriptional regulates a number of DNA repair genes, but that in cells transfected with siRNA targeted to FOXM1 had increased DNA breaks, too [115]. In their study, Tan and colleagues also showed that these FOXM1-deficient cells increased activation of the tumour suppressor, p53, thus identifying a role for FOXM1 in the transcriptional response during DNA damage/checkpoint signalling [115].

As discussed earlier, Fang and colleagues observed that Thiostrepton enhanced DNA damage and PARP1 trapping in cells treated with Olaparib [310]. In their

study, they observed an increased level of γH2AX with Thiostrepton treatment, and this increase was enhanced when combined with Olaparib [310]. The data presented by Fang and colleagues in ovarian cancer cells corroborates our data in breast cancer cells, as we also observed an increased level of γH2AX in cells treated with Thiostrepton and Olaparib [310]. This significant increase in γH2AX foci in our study again implicates FOXM1 as playing an important role in regulating DNA repair genes and maintaining DNA repair efficacy.

Furthermore, as γH2AX were observed in cells treated after 24-hours with our agents, this could account for the increased RAD51 protein expression initially observed in our Thiostrepton time course treatment Western blot, and could explain why this trend was not observed at the mRNA expression level. Thus still implicating RAD51 as playing a more prominent role in mediating sensitisation to Olaparib, after Thiostrepton treatment.

Measuring γH2AX has clinical applications such as assessing DSBs levels induced by radio- and chemotherapy as a marker of treatment efficacy [345]. It is also used in dose/scheduling estimation, as well as in determining the efficiency of DNA repair to predict potential tumour sensitivity or resistance to DNA damaging anticancer agents [345]. Furthermore, by determining DSB levels induced by anticancer treatment in normal cells, this may help in predicting toxicity of anticancer treatment [345]. The main advantage of this as an approach, compared to other DNA damage markers, is the high sensitivity of the technique [345]. That said, other considerations include background levels of foci, and could make this difficult in predicting the toxicity of anticancer treatments in patients, when comparing DSBs in tumour cells and normal cells [345].

1.6 What about other combination therapies?

Our research has clearly demonstrated that Thiostrepton and Olaparib as a combination therapy could be further pursued clinically, and could subsequently offer breast cancer patients an alternative treatment option. That said, this by no means excludes the exploration of other potential treatment avenues, which may ultimately build on our study.

Interestingly, a recent study has uncovered that PARP inhibitors not only cause tumour cell death by damaging their DNA, these cells are also subjected to attack by immune cells that are attracted to them [346]. PARP inhibitors seemingly unmask cancer cells from evasion of immune cells, and could help make immunotherapy work in this patient population [346]. The authors also discuss how there are clinical trials combining PARP inhibitors with anti-PD-L1 in *Brca1*/*2*-mutant breast and ovarian cancer, and that other histologies may also benefit, such as ERCC1-deficient non–small cell lung cancer [346]. Therefore, in the context of our study it could be possible to further enhance the response of breast cancer cells to Thiostrepton and Olaparib by combining this with anti-PD-L1 therapy.

Recent advances suggest that targeting both the tumour cell and its interaction with the immune microenvironment may significantly improve patient benefit. To this end, inhibitors of the immune checkpoint molecules PD-1, CTLA-4, and PD-L1 have recently shown great clinical promise and represent a novel way of treating cancer [347]. To date, one of the few genetic determinants of response to immune checkpoint blockade identified is the presence of mutations in DNA repair genes [348, 349]. It was hypothesised that this was due to the increased amount of somatic mutations in DNA repair-deficient tumours, which can encode “non-self”

immunogenic antigens [348]. A number of clinical studies have identified the presence of mutations in DNA repair genes in patients who have responded to checkpoint inhibitors. A recent study indicated that MMR status predicted clinical benefit with the PD-1 inhibitor, Pembrolizumab [349]. In addition, mutations in the DNA repair and replication genes POLD1, POLE, Lig3, RAD17, and BRCA2, were identified in melanoma and lung cancer patients that had a better clinical response to PD-1 inhibition [350]. Given that we have observed a decrease in a range of DNA repair genes and an increase in DNA double-strand breaks upon FOXM1 inhibition, it would be interesting to understand whether a combination therapy of Thiostrepton with an immune checkpoint inhibitor may have clinical benefit.

As DNA damage has represented a backbone of cancer treatment, other combinations may also be considered. Tumours with HR deficiencies would be synthetically lethal with other DNA repair inhibitors, not just PARP inhibitors. As Minchom and colleagues discuss, there are ATM, ATR and CHK1 inhibitors, and these are being tested in clinical trials [351]. The benefit of such alternative options could be used, for example, in patients who may become resistant to PARP inhibitors. In one study, when compared with BRCA1-proficient cells, PARP inhibitor-resistant BRCA1-deficient cells were shown to be increasingly dependent on ATR for survival [352]. So, the authors suggest that ATR inhibition can be used to overcome PARP inhibitor resistance in BRCA-deficient cancers [352]. In the case of our study, the implication of this is that Thiostrepton may ultimately synergise to cause synthetic lethality with other inhibitors targeted against DNA damage response defects in the cells. Furthermore, if resistance to PARP inhibitors occurred in patients treated with our combination therapy, other inhibitors such as ATR inhibitors may become more relevant in this particular clinical setting.

Taken together, we have shown for the first time that Thiostrepton and Olaparib in combination caused greater reduction in cell viability and colony formation, compared to either agent alone, in breast cancer cells. Furthermore, this response was not FOXM1- or RAD51-expression level dependent. As Olaparib has already been approved for clinical use, Thiostrepton would be the focus to accelerate this combination therapy into the clinic. This would require development of Thiostrepton for use in humans, and then for use in combination with Olaparib. We have also investigated the mechanistic implications of our combination therapy in breast cancer cells. Our data has suggested that the HR proteins RAD51 and BRIP1 may be playing an important role in mediating Olaparib sensitisation, upon treatment with Thiostrepton. Furthermore, our data using non-tumourigenic breast epithelial cells has suggested a possible therapeutic window, where short-term Thiostrepton exposure with subsequent Olaparib sensitisation could be the most effective way to treat breast cancer patients, and to spare their normal cells. Finally, increased DNA damage was observed in breast cancer cells treated with our combination therapy, compared to either agent alone, suggesting that this may be a mechanistic basis for these compounds acting together. We also discussed how a combination therapy of Thiostrepton with an immune checkpoint inhibitor may benefit patients, given our data showing how Thiostrepton treatment caused decreased DNA repair genes and increased DNA damage. Overall, Thiostrepton and Olaparib may be considered as a combination therapy with clinical relevance for treating cancer patients.

2.0 Thiostrepton and Diminutol are synthetically lethal in ovarian cancer

2.1 FOXM1 and oxidative stress

FOXM1 also plays a role in regulating the oxidative stress response [119, 120]. Cancer cells that overexpress FOXM1 can harness this role during initiation and progression of the tumour. Whilst we have already discussed a potentially viable treatment strategy for targeting FOXM1 and homologous recombination, we also wanted to explore the possibility of developing another combination therapy option, by exploiting a different pathway regulated by FOXM1. Given the emergence of resistance to treatment is common for cancer patients, this emphasises the need to investigate novel therapies in a range of different strategies. This project therefore next proposed to exploit the role of FOXM1 in oxidative stress, by initially determining the relationship between FOXM1 and members of the oxidative stress response.

The role of FOXM1 in regulating oxidative stress has been outlined in the introduction, such as transcriptional regulation of antioxidant genes MnSOD and catalase [119]. Furthermore, ROS levels have been shown to be elevated in FOXM1-deficient cells, compared to normal cells, emphasising FOXM1's role as an important regulator of intracellular ROS [119]. Therefore, based on the current data, this led us to further explore the role of FOXM1 in regulating the oxidative stress response.

We began by determining how expression levels of proteins involved in the oxidative stress response changed upon *Foxm1* silencing, in breast cancer cell

lines. This data subsequently formed the basis for the rest of our study. We were able to observe a consistent decrease in *Nrf2* mRNA expression upon *Foxm1* silencing, which validated previous work that FOXM1 regulates NRF2 expression [302, 303]. Notably, we also observed an increase in the expression of NQO1, which is downstream of NRF2, upon *Foxm1* silencing, and this expression change became the focus of our later investigations, because the relationship between FOXM1 and NQO1 had also not yet been fully evaluated.

2.2 Potential role for FOXM1 and NQO1 in mediating chemoresistance

Since chemoresistance plays a large role in hindering a patient's response to treatment, it is essential to assess which mechanisms cancer cells may be using to cause chemoresistance, and to in turn discover new ways to target this in cancer cells. As previously mentioned, both FOXM1 and NQO1 have been implicated in chemoresistance [188, 320].

Given that we observed an increase in NQO1 expression upon FOXM1 loss, we aimed to understand whether FOXM1 and NQO1 were involved in a co-ordinated response to chemotherapy. To investigate this further, we used a model of chemo-sensitivity and –resistance, and this was in the form of platinum-sensitive and –resistant ovarian cancer cell lines. Once platinum sensitivity had been validated, we indeed observed that our platinum-resistant ovarian cancer cell lines had higher expression levels of both FOXM1 and NQO1, compared to our platinum-sensitive cell line. This confirmed published data that these proteins are often upregulated in chemoresistant cancer cells. In Docetaxel-resistant human lung adenocarcinoma

A549 cells FOXM1 has been shown to be upregulated, compared to the parental cells [321]. Furthermore, in Cisplatin-resistant A2780cp cells, NRF2 and NQO1 have both been shown to be upregulated, compared to the parental cells [353].

Significantly, when *Nqo1* was silenced, we saw a corresponding decrease in FOXM1 expression levels. We also confirmed what we initially observed in breast cancer cells, that when *Foxm1* was silenced, we saw a corresponding increase in NQO1 expression levels. This result was further confirmed using a different approach to siRNA, where treatment with the FOXM1 inhibitor Thiostrepton also showed an increase in NQO1 expression. These data suggest that FOXM1 and NQO1 expression are potentially regulated by each other, and may have compensatory functional roles in the cell. Whilst there seems to be no evidence in the literature suggesting a direct link between FOXM1 and NQO1, these changes in protein expression could be occurring indirectly. Since NRF2 is a transcriptional target of FOXM1, and NQO1 is expressed by NRF2 regulation, this indicates that there could be a link between FOXM1 and NQO1 through the NRF2 pathway [302, 303].

Since our data thus far has suggested that FOXM1 and NQO1 may be acting together in cells, we next investigated this relationship in a Cisplatin-treatment setting. In this instance, both FOXM1 and NQO1 expression levels were shown to be upregulated after cells were exposed to Cisplatin, and this was independent of chemoresistance of the cells (Figure 24). It is also important to note that when Kwok and colleagues investigated acquired Cisplatin resistance in breast cancer cells, they assessed the contribution of FOXM1 and downstream targets BRCA2 and XRCC1, and discussed how FOXM1 is not the sole regulator of these genes, and

that additional roles of FOXM1 may add to causing Cisplatin resistance [188]. Our data showed that silencing *Nqo1* did not re-sensitize platinum-resistant cells to Cisplatin, therefore suggesting that NQO1 is not the sole mediator of chemoresistance in these cells and may have a co-ordinated role with other FOXM1-target proteins such as BRCA2 and XRCC1 in maintaining resistance to these drugs. We did, however, observe increased resistance to Cisplatin when *Nqo1* was silenced in Cisplatin-sensitive cells only. Therefore, this led us to hypothesise that FOXM1 and NQO1 may play a role together in causing chemoresistance in chemosensitive cells.

Whilst our data so far had evaluated the roles of FOXM1 and NQO1 individually in chemoresistance, we also wanted to assess whether these proteins acted together in chemoresistance. In one study, a link between FOXM1 and the antiapoptotic genes, XIAP and Survivin, in the modulation of chemoresistance in breast cancer cells was established [191]. The authors were able to show that FOXM1 transcriptionally activated XIAP and Survivin expression [191]. This type of study shows that different pathways can converge on generating a chemoresistant phenotype and our study may show a different mechanism of how two proteins may act together to mediate chemoresistance, where the downstream effector is not necessarily a direct target of the upstream regulator.

Our data suggested that silencing *Foxm1* and *Nqo1* silencing together caused the greatest reduction in cell viability, and this was again in platinum-sensitive cells, rather than platinum-resistant cells. The implications of this could be that FOXM1 and NQO1 have a threshold of expression for cell viability, and because platinum-sensitive cells have lower expression levels of these proteins they are increasingly

sensitive to their inhibition. This suggests that upon silencing of *Foxm1* and *Nqo1* in platinum-resistant cells, where their expression is higher, that the remaining levels of FOXM1 and NQO1 are sufficient for viability.

When *Foxm1* and *Nqo1* were individually silenced, re-sensitisation to Cisplatin was not observed so we next addressed whether *Foxm1* and *Nqo1* silencing together would lead to Cisplatin re-sensitisation. This approach has been similarly investigated in studies where FOXM1 and downstream targets of FOXM1 have been implicated in chemoresistance [116, 308]. In recurrent glioblastoma cells, Zhang and colleagues showed that targeting FOXM1 was shown to sensitise resistant cells to Temozolomide, by downregulating RAD51 expression [116]. Furthermore, in ovarian cancer cells, Zhou and colleagues showed that targeting FOXM1 and its target gene EXO1 could increase sensitivity to Cisplatin [308]. These studies have illustrated that FOXM1 is not acting alone in chemoresistance, and that other proteins may be playing a role in generating a chemoresistant phenotype. However, we did not observe re-sensitisation to Cisplatin when *Foxm1* and *Nqo1* were silenced together.

Interestingly, whilst NRF2 has been regarded as the main transcriptional regulator of NQO1, there are studies showing there are other regulators of NQO1 transcription. In a study where ROS and the pathogenesis of Dox-induced cardiomyopathy were investigated, an NRF2-independent activation of NQO1 was identified, where Progesterone was shown to induce NQO1 [354]. This study therefore indicates that other pathways could be involved in regulating NQO1 expression, in relation to changes in FOXM1 expression, when considering their relationship and their roles in chemoresistance.

Having assessed the response of our cells to Cisplatin when *Foxm1* and *Nqo1* were silenced together, we next treated our cells with the FOXM1 inhibitor Thiostrepton, so that an alternate approach to using siRNA was tested. Our data supported previously published data that had shown synergy between Thiostrepton and Cisplatin, and we observed a combined action that was greater in both Cisplatin-sensitive and -resistant cells [188, 308, 309]. In the study by Kwok and colleagues, they discussed how Thiostrepton synergised with Cisplatin and reversed acquired Cisplatin-resistance in breast cancer cells, and caused increased Cisplatin-induced cell death [188].

Whilst the main focus of our study was on FOXM1 and NQO1, we did also investigate other members of the NRF2 pathway, to gain a more detailed understanding of the role of these proteins and potentially other expression changes upon chemoresistance. In particular, we observed that the antioxidant response protein, SOD1 was upregulated in platinum-resistant ovarian cancer cells, compared to the platinum-sensitive parental ovarian cancer cells (Figure 30). We also observed an increased expression of Gpx1, upon *Nqo1* silencing and upon *Foxm1* + *Nqo1* silencing, in Carboplatin-resistant cells. We therefore suggested that FOXM1 and NQO1 may be regulating the NRF2-antioxidant pathway in general. Interestingly, overexpression of SOD1 has already been implicated in causing Cisplatin resistance in ovarian cancer cells, thus supporting SOD1 as another potential target for a novel anticancer chemosensitiser [355]. Furthermore, in a study of pancreatic ductal adenocarcinoma, Gpx1 silencing resulted in the epithelial–mesenchymal transition and Gemcitabine resistance [356]. It was also shown that Gpx1 expression negatively correlated with pancreatic ductal adenocarcinoma prognosis [356].

2.3 Selecting compounds for our combination therapy

Our novel data accumulated so far suggests that both FOXM1 and NQO1 may be playing a role in mediating chemoresistance, because they are upregulated in chemoresistant cells, their expression levels change when the other changes, and there seems to be a threshold of expression in platinum-sensitive cells. This next raised the possibility of developing a combination treatment strategy to target these proteins, in cancer cells. This strategy could also hold further implications; such that is could potentially reduce the risk of cancer cells becoming chemoresistant.

From the outset, we wanted to investigate the relationship between FOXM1 and members of the oxidative stress response. We consequently found that a relationship existed between FOXM1 and NQO1, and that this could be playing a role in mediating chemoresistance of cancer cells. We next investigated a synthetic lethal approach, through exploiting the role of FOXM1 in oxidative stress. We discovered that it may be possible to target FOXM1 and the antioxidant protein NQO1, in cancer cells.

NQO1 inhibitors may represent ideal candidates to be used alongside the FOXM1 inhibitor, Thiostrepton. Notably, the NQO1 inhibitor Dicoumarol could be argued as the best candidate for our study, because of it being the most commonly used NQO1 inhibitor and it has a high potency. That said, as previously highlighted, there have been a number of studies that have revealed problems with using Dicoumarol as an NQO1 inhibitor, including mitochondrial uncoupling [160].

Other Coumarin-based compounds that were reviewed in the introduction, as well as another competitive inhibitor of NQO1, Diminutol, could have offered the potential to be used in our combination therapy treatment strategy. Since we were not evaluating the activating or detoxifying properties of NQO1, we did not use irreversible inhibitors for our study. Furthermore, in the case of Coumarin-based compounds, whilst they seemingly showed greater potency than Dicoumarol, they were not without their limitations [162, 164, 165]. In particular, a problem that was also identified with Dicoumarol is that protein binding could compromise the activity of these compounds [162]. This was tested in the presence and absence of BSA, and was found to be variable between compounds [162]. Therefore, we selected Diminutol due to the novelty of this compound as an inhibitor of NQO1, as there has been limited investigation of this compound in current literature. So, we were therefore keen to assess the mechanistic implications of this compound in our combination therapy, when treating cancer cells.

We were able to show that Thiostrepton and Diminutol acted in combination in ovarian cancer cells, using cell viability assays. Our results indicated that FOXM1 inhibition using Thiostrepton enhanced sensitivity to the NQO1 inhibitor Diminutol, as shown by our experimental approach. This also seemed to be independent of having lower FOXM1 and/or NQO1 expression levels, therefore having the potential to be used to treat more patients. Taken together, our combination therapy data has revealed a novel strategy for targeting FOXM1 and the oxidative stress response in cancer cells, through targeting NQO1. This is the first time that targeting NQO1 has been used in a synthetic lethal approach with targeting FOXM1, as previous studies have shown that synergy could be achieved in cancer cells treated with FOXM1 inhibitors in combination with ROS inducers [187].

The benefits of our combination therapy, of Thiostrepton and Diminutol, compared to current standard of care treatments, is that we have developed a novel treatment option that may perturb the emergence of chemoresistance which has delayed successful responses to therapy. This type of approach may be appropriate for platinum-resistant patients, those who have relapsed within six months of completing first-line therapy and may have less than 15% response rates to subsequent chemotherapy [357]. In these cases, their progression-free survival could be 3-4 months and their median survival less than a year [357].

To be pursued clinically, there may be more obstacles to overcome, compared to those outlined in the case of our Thiostrepton and Olaparib combination therapy. Whilst we have initially viewed Diminutol as an ideal compound to be used in combination with Thiostrepton, because of the novelty aspect, this may in fact hinder clinical progression. A lack of clinical data for Diminutol would make this more of a priority in the first instance so that an assessment of how this compound may be used in the treatment of cancer patients could then be investigated in a clinical setting. Once this has been established, other factors regarding time- and dose-specific considerations can be evaluated in cancer cells and patients. Safety and efficacy of Diminutol include some of the main profiles that will need to be assessed before this compound can be used in human cancer treatment [358].

2.4 Exploring the mechanism of Thiostrepton and Diminutol as a combination therapy

Once our combination therapy had been established we then decided to explore the mechanistic implications of this, by assessing γH2AX and ROS levels in cells

treated with single agents and our agents in combination. A study has shown that cells transfected with siRNA targeted to FOXM1 had increased DNA breaks [115]. Furthermore, we showed an increased level of γH2AX with Thiostrepton treatment, and this increase was enhanced when combined with Olaparib, in breast cancer cells. Based on this, we therefore first investigated γH2AX foci in ovarian cancer cells treated with agents alone and agents in combination.

Our results showed that our Cisplatin-resistant cells had increased γH2AX foci when treated with Thiostrepton and Diminutol in combination, compared to either agent alone. As suggested in our results, it could be that increased DSBs may be contributing to the mechanism of action of our combination therapy, where other actions may also be contributing to the overall mechanism. In a study which included the investigation of the response of breast cancer cells to ROS-inducing β-lapachone treatment, the observed cellular changes were the generation of NQO1-dependent ROS, DNA breaks, Ca^{2+}-dependent γH2AX formation and PARP-1 hyperactivation [359]. Such studies demonstrate that further mechanistic implications may be involved in mediating the combined activity between FOXM1 inhibition and NQO1 inhibition.

Subsequently, we next investigated ROS levels in our cells treated with Thiostrepton alone, Diminutol alone, and the agents in combination. Our Cisplatin-resistant cells had increased ROS levels when treated with Thiostrepton and Diminutol in combination, compared to either agent alone. This was not observed in our platinum-sensitive cells. Previously, FOXM1-deficiency has shown elevated ROS in the human primary fibroblasts IMR90, while Thiostrepton treatment has shown increased ROS in melanoma cells [119, 326]. Furthermore, in lung

adenocarcinoma cells depleted of NQO1, there was increased endogenous ROS [327], and pancreatic cancer cells treated with the NQO1 inhibitor, Dicoumarol, induced growth inhibition through increased ROS production [328]. Conversely, prostate cancer cells had decreased endogenous ROS after inhibition of NQO1 [329], and cholangiocarcinoma cells treated with the NQO1 inhibitor Dicoumarol showed no increase in ROS formation [158]. These differences in ROS generation were suggested to be cell-specific, such that NQO1 inhibition could exert different cell killing mechanisms. Furthermore, regulation through the ROS response may be selective. Therefore, in the context of our results, it may be suggested that Cisplatin-resistant cells are sensitised to our combination therapy, through increased ROS generation, compared to platinum-sensitive cells.

2.5 Other combination therapy options, FOXM1 inhibitors and ROS inducers

In the final part of our project we were able to corroborate the findings by Halasi and colleagues, who successfully showed that oxidative stress and FOXM1 inhibitors could be combined to induce cell death in cancer cells [187]. Halasi and colleagues used ROS inducers including β-phenylethyl isothiocyanate (PEITC) and 2-methoxyestradiaol (2-ME) in different human cancer cells [187]. Both of these compounds were tested in this study because they are being used in clinical trials, where 2-ME was used to confirm the effects of PEITC [187]. Therefore, the authors were able to show that suppression of FOXM1 further sensitised cancer cells to oxidative stress–mediated apoptosis induced by different types of ROS inducers [187].

Using the FOXM1 inhibitor Thiostrepton in combination with the ROS inducer Menadione, or Podophyllotoxin, we validated what was observed in the study by Halasi and colleagues, using different ROS inducers to those used in their study [187]. Therefore, we have shown for the first time that Menadione and Podophyllotoxin with Thiostrepton caused a greater reduction in cell viability and colony formation, respectively, than either agent alone, in breast cancer cells.

Notably, Halasi and colleagues also included *in vivo* work to further support their findings. They showed that the FOXM1 inhibitior Bortezomib in combination with the ROS inducer β-phenylethyl isothiocyanate efficiently inhibited the growth of breast tumour xenografts in nude mice [187]. The authors discussed how this combination therapy may be less toxic to normal cells because of a generally lower expression of FOXM1 and less dependence of their antioxidant system, compared to tumour cells [187]. It would be beneficial, especially in consideration of our novel combination therapy of Thiostrepton and Diminutol, to test this combination in *in vivo* studies, to further develop and support *in vitro* investigations that we have already carried out in our project.

Taken together, our data shows that FOXM1 and NQO1 may play a role in chemoresistance in ovarian cancer cells. We have suggested that NQO1 is not the sole mediator of chemoresistance in these cells and may have a co-ordinated role with other FOXM1-target proteins such as BRCA2 and XRCC1 in maintaining resistance to these drugs. Loss of FOXM1 and NQO1, using siRNA, resulted in a greater reduction in cell viability in chemosensitive ovarian cancer cells, suggesting that these cells may have a threshold of expression of these proteins that is required for viability. We have also shown for the first time that Thiostrepton and

Diminutol in combination caused a greater reduction in cell viability, compared to either agent alone, in ovarian cancer cells. Furthermore, this response was not FOXM1- or NQO1-expression level dependent. Interestingly, the novelty of Diminutol as an NQO1 inhibitor is what made it an attractive compound to use in our studies, however to be pursued clinically this compound would require further study. We have also investigated the mechanistic implications of our combination therapy in ovarian cancer cells. Our data suggested that increased DNA damage in ovarian cancer cells treated with our combination therapy, compared to either agent alone, and increased levels of ROS may both contribute to a mechanistic basis for the cellular response. Overall, Thiostrepton and Diminutol may be considered as a combination therapy with clinical relevance for treating cancer patients.

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